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I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): **Paul J. Godowski**
 Austin L. Gurney

For: **TIE LIGANDS**

1. **Type of Application**
This new application is for a(n) x original _ continuation-in-part application.

2. **Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b)(Regular)**
 89 pages of specification
 3 pages of claims
 1 page(s) of abstract
 25 sheet(s) of drawings
 [x] formal [] informal

3. **Declaration or Oath**
 X The declaration of the inventor(s) [x] is enclosed []unsigned [] will follow.

4. **Assignment**
 X An Assignment of the invention to GENENTECH, INC. [x] is enclosed with attached
Recordation Form Cover Sheet [] will follow.

5. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

| CLAIMS FOR FEE CALCULATION | | | | | |
|--|----|--------------|---|------------|-----------------------------|
| Number Filed | | Number Extra | | Rate | Basic Fee 37 CFR 1.16(a) |
| | | | | | \$770.00 |
| Total Claims | 25 | - 20 = | 5 | X \$22.00 | \$110.00 |
| Independent Claims | 2 | - 3 = | 0 | X \$80.00 | \$0.00 |
| <u>x</u> Multiple dependent claim(s), if any | | | | + \$260.00 | \$260.00 |
| Filing Fee Calculation | | | | | \$1140.00 |

6. Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$1,140.00. **A duplicate copy of this transmittal is enclosed.**

7. Authorization to Charge Additional Fees

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

8. Additional Papers Enclosed

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☒ Submission of "Sequence Listing", computer readable copy and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ Other:

Respectfully submitted,
GENENTECH, INC.

Date: September 19, 1997By: 

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PATENT DOCKET P1130

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TIE LIGANDS

Field of the Invention

The present invention concerns isolated nucleic acid molecules encoding novel TIE ligands, the TIE proteins encoded by such nucleic acid molecules, as well as methods and means for making and using such nucleic acid and protein molecules.

Background Art

The abbreviations "TIE" or "tie" are acronyms, which stand for "tyrosine kinase containing Ig and EGF homology domains" and were coined to designate a new family of receptor tyrosine kinases which are almost exclusively expressed in vascular endothelial cells and early hemopoietic cells, and are characterized by the presence of an EGF-like domain, and extracellular folding units stabilized by intra-chain disulfide bonds, generally referred to as "immunoglobulin (IG)-like" folds. A tyrosine kinase homologous cDNA fragment from human leukemia cells (tie) was described by Partanen et al., Proc. Natl. Acad. Sci. USA 87, 8913-8917 (1990). The mRNA of this human "tie" receptor has been detected in all human fetal and mouse embryonic tissues, and has been reported to be localized in the cardiac and vascular endothelial cells. Korhonen et al., Blood 80, 2548-2555 (1992); PCT Application Publication No. WO 93/14124 (published 22 July 1993). The rat homolog of human tie, referred to as "tie-1", was identified by Maisonpierre et al., Oncogene 8, 1631-1637 (1993)). Another tie receptor, designated "tie-2" was originally identified in rats (

Dumont et al., Oncogene 8, 1293-1301 (1993)), while the human homolog of tie-2, referred to as "ork" was described in U.S. Patent No. 5,447,860 (Ziegler). The murine homolog of tie-2 was originally termed "tek." The cloning of a mouse tie-2 receptor from a brain capillary cDNA library is disclosed in PCT Application Publication No. WO 95/13387 (published 18 May 1995). The TIE receptors are believed to be actively involved in angiogenesis, and may play a role in hemopoiesis as well.

The expression cloning of human TIE-2 ligands has been described in PCT Application Publication No. WO 96/11269 (published 18 April 1996) and in U.S. Patent No. 5,521,073 (published 28 May 1996). A vector designated as λ gt10 encoding a TIE-2 ligand named "htie-2 ligand 1" or "hTL1" has been deposited under ATCC Accession No. 75928. A plasmid encoding another TIE-2 ligand designated "htie-2 2" or "hTL2" is available under ATCC Accession No. 75928. This second ligand has been described as an antagonist of the TAI-2 receptor. The identification of secreted human and mouse ligands for the TIE-2 receptor has been reported by Davis et al., Cell 87, 1161-1169 (1996). The human ligand designated "Angiopoietin-1", to reflect its role in angiogenesis and potential action during hemopoiesis, is the same ligand as the ligand variously designated as "htie-2 1" or "hTL-1" in WO 96/11269. Angiopoietin-1 has been described to play an angiogenic role later and distinct from that of VEGF (Suri et al., Cell 87, 1171-1180 (1996)). Since TIE-2 is apparently upregulated during the pathologic angiogenesis requisite for tumor growth (Kaipainen et al., Cancer Res. 54, 6571-6577 (1994)) angiopoietin-1 has been suggested to be additionally useful for specifically targeting tumor vasculature (Davis et al., supra).

Summary of the Invention

The present invention concerns novel human TIE ligands with powerful effects on vasculature. The invention also provides for isolated nucleic acid molecules encoding such ligands or functional derivatives thereof, and vectors containing such nucleic acid molecules. The invention further concerns host cells transformed with such nucleic acid to produce the novel TIE ligands or functional derivatives thereof. The novel ligands may be agonists or antagonists of TIE receptors, known or hereinafter discovered. Their therapeutic or diagnostic use, including the delivery of other

therapeutic or diagnostic agents to cells expressing the respective TIE receptors, is also within the scope of the present invention.

The present invention further provides for agonist or antagonist antibodies specifically binding the TIE ligands herein, and the diagnostic or therapeutic use of such antibodies.

5 In another aspect, the invention concerns compositions comprising the novel ligands or antibodies.

In a further aspect, the invention concerns conjugates of the novel TIE ligands of the present invention with other therapeutic or cytotoxic agents, and compositions comprising such conjugates. Because the TIE-2 receptor has been reported to be upregulated during the pathologic angiogenesis that is requisite for tumor growth, the conjugates of the TIE ligands of the present invention to
10 cytotoxic or other anti-tumor agents are useful in specifically targeting tumor vasculature.

In yet another aspect, the invention concerns a method for identifying a cell that expresses a TIE (e.g. TIE-2) receptor, which comprises contacting a cell with a detectably labeled TIE ligand of the present invention under conditions permitting the binding of such TIE ligand to the TIE
15 receptor, and determining whether such binding has indeed occurred.

In a different aspect, the invention concerns a method for measuring the amount of a TIE ligand of the present invention in a biological sample by contacting the biological sample with at least one antibody specifically binding the TIE ligand, and measuring the amount of the TIE ligand-antibody complex formed.

20 The invention further concerns a screening method for identifying polypeptide or small molecule agonists or antagonists of a TIE receptor based upon their ability to compete with a native or variant TIE ligand of the present invention for binding to a corresponding TIE receptor.

The invention also concerns a method for imaging the presence of angiogenesis in wound healing, in inflammation or in tumors of human patients, which comprises administering detectably
25 labeled TIE ligands or agonist antibodies of the present invention, and detecting angiogenesis.

In another aspect, the invention concerns a method of promoting or inhibiting neovascularization in a patient by administering an effective amount of a TIE ligand of the present

invention in a pharmaceutically acceptable vehicle. In a preferred embodiment, the present invention concerns a method for the promotion of wound healing. In another embodiment, the invention concerns a method for promoting angiogenic processes, such as for inducing collateral vascularization in an ischemic heart or limb. In a further preferred embodiment, the invention

5 concerns a method for inhibiting tumor growth.

In yet another aspect, the invention concerns a method of promoting bone development and/or maturation and/or growth in a patient, comprising administering to the patient an effective amount of a TIE ligand of the present invention in a pharmaceutically acceptable vehicle.

In a further aspect, the invention concerns a method of promoting muscle growth and development, which comprises administering a patient in need an effective amount of a TIE ligand

10 of the present invention in a pharmaceutically acceptable vehicle.

The TIE ligands of the present invention may be administered alone, or in combination with each other and/or with other therapeutic or diagnostic agents, including members of the VEGF family. Combinations therapies may lead to new approaches for promoting or inhibiting neovascularization, and muscle growth and development.

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Brief Description of the Figures

Figure 1-A is the nucleotide sequence of FLS139 (SEQ. ID. NO.: 16).

Figure 1-B is the amino acid sequence of FLS139 (SEQ. ID. NO.: 17).

Figure 2 is the nucleotide sequence of the TIE ligand NL1 (SEQ. ID. NO: 1) (DNA 22779).

Figure 3 is the amino acid sequence of the TIE ligand NL1 (SEQ. ID. NO:2).

Figure 4 is the nucleotide sequence of the TIE ligand NL5 (SEQ. ID. NO: 3) (DNA 28497).

Figure 5 is the amino acid sequence of the TIE ligand NL5 (SEQ. ID. NO: 4).

Figure 6 is the nucleotide sequence of the TIE ligand NL8 (SEQ. ID NO: 5) (DNA 23339).

Figure 7 is the amino acid sequence of the TIE ligand NL8 (SEQ. ID NO:6).

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Figures 8-A and 8-B show the expression of NL1 in various tissues as determined by *in situ* hybridization to cellular RNA.

Figures 9-A and 9-B show the expression of NL5 in various tissues as determined by *in situ* hybridization to cellular RNA.

Figures 10-A and 10-B show the expression of NL8 in various tissues as determined by *in situ* hybridization to cellular RNA.

Figure 11 and 12 - Northern blots showing the expression of the mRNAs of TIE ligands NL1 and NL5 in various tissues.

Detailed Description of the Invention

A. TIE LIGANDS AND NUCLEIC ACID MOLECULES ENCODING THEM

The TIE ligands of the present invention include the native human ligands designated NL1 (SEQ. ID. NO: 2), NL5 (SEQ. ID. NO: 4), and NL8 (SEQ. ID. NO: 6), their homologs in other, non-human mammalian species, including, but not limited to, higher mammals, such as monkey; rodents, such as mice, rats, hamster; porcine; equine; bovine; naturally occurring allelic and splice variants, and biologically active (functional) derivatives, such as, amino acid sequence variants of such native molecules, as long as they differ from a native TL-1 or TL-2 ligand. The native TIE ligands of the present invention are substantially free of other proteins with which they are associated in their native environment. This definition is not limited in any way by the method(s) by which the TIE ligands of the present invention are obtained, and includes all ligands otherwise within the definition, whether purified from natural source, obtained by recombinant DNA technology, synthesized, or prepared by any combination of these and/or other techniques. The amino acid sequence variants of the native TIE ligands of the present invention shall have at least about 90%, preferably, at least about 95%, more preferably at least about 98%, most preferably at least about 99% sequence identity with a full-length, native human TIE ligand of the present invention, or with the fibrinogen-like domain of a native human TIE ligand of the present invention. Such amino acid sequence variants preferably exhibit or inhibit a qualitative biological activity of a native TIE ligand.

The term "fibrinogen domain" or "fibrinogen-like domain" is used to refer to amino acids from about position 278 to about position 498 in the known hTL-1 amino acid sequence; amino acids

from about position 276 to about position 496 in the known hTL-2 amino acid sequence; amino acids from about position 270 to about 493 in the amino acid sequence of NL1; amino acids from about position 272 to about position 491 in the amino acid sequence of NL5; and amino acids from about position 252 to about position 470 in the amino acid sequence of NL8; and to homologous domains in other TIE ligands.

The term “nucleic acid molecule” includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given TIE ligand may be produced. The present invention specifically contemplates every possible variation of nucleotide sequences, encoding the TIE ligands of the present invention, based upon all possible codon choices. Although nucleic acid molecules which encode the TIE ligands herein are preferably capable of hybridizing, under stringent conditions, to a naturally occurring TIE ligand gene, it may be advantageous to produce nucleotide sequences encoding TIE ligands, which possess a substantially different codon usage. For example, codons may be selected to increase the rate at which expression of the polypeptide occurs in a particular prokaryotic or eukaryotic host cells, in accordance with the frequency with which a particular codon is utilized by the host. In addition, RNA transcripts with improved properties, e.g. half-life can be produced by proper choice of the nucleotide sequences encoding a given TIE ligand.

“Sequence identity” shall be determined by aligning the two sequences to be compared following the Clustal method of multiple sequence alignment (Higgins et al., Comput. Appl. Biosci. 5, 151-153 (1989), and Higgins et al., Gene 73, 237-244 (1988)) that is incorporated in version 1.6 of the Lasergene biocomputing software (DNASTAR, Inc., Madison, Wisconsin), or any updated version or equivalent of this software.

The terms “biological activity” and “biologically active” with regard to a TIE ligand of the present invention refer to the ability of a molecule to specifically bind to and signal through a native TIE receptor, e. g. a native TIE-2 receptor, or to block the ability of a native TIE receptor (e.g. TIE-2) to participate in signal transduction. Thus, the (native and variant) TIE ligands of the present invention include agonists and antagonists of a native TIE, e.g. TIE-2, receptor. Preferred biological activities of the TIE ligands of the present invention include the ability to induce or inhibit

vascularization. The ability to induce vascularization will be useful for the treatment of biological conditions and diseases, where vascularization is desirable, such as wound healing, ischaemia, and diabetes. On the other hand, the ability to inhibit or block vascularization may, for example, be useful in preventing or attenuating tumor growth. Another preferred biological activity is the ability to affect muscle growth or development. A further preferred biological activity is the ability to influence bone development, maturation, or growth.

The term “functional derivative” is used to define biologically active amino acid sequence variants of the native TIE ligands of the present invention, as well as covalent modifications, including derivatives obtained by reaction with organic derivatizing agents, post-translational modifications, derivatives with nonproteinaceous polymers, and immunoadhesins.

“Vascular endothelial growth factor”/“vascular permeability factor” (VEGF/VPF) is an endothelial cell-specific mitogen which has recently been shown to be stimulated by hypoxia and required for tumor angiogenesis (Senger et al., Cancer **46**: 5629-5632 (1986); Kim et al., Nature **362**:841-844 (1993); Schweiki et al., Nature **359**: 843-845 (1992); Plate et al., Nature **359**: 845-848 (1992)). It is a 34-43 kDa (with the predominant species at about 45 kDa) dimeric, disulfide-linked glycoprotein synthesized and secreted by a variety of tumor and normal cells. In addition, cultured human retinal cells such as pigment epithelial cells and pericytes have been demonstrated to secrete VEGF and to increase VEGF gene expression in response to hypoxia (Adamis et al., Biochem. Biophys. Res. Commun. **193**: 631-638 (1993); Plouet et al., Invest. Ophthalmol. Vis. Sci. **34**: 900 (1992); Adamis et al., Invest. Ophthalmol. Vis. Sci. **34**: 1440 (1993); Aiello et al., Invest. Ophthalmol. Vis. Sci. **35**: 1868 (1994); Simorre-pinatel et al., Invest. Ophthalmol. Vis. Sci. **35**: 3393-3400 (1994)). In contrast, VEGF in normal tissues is relatively low. Thus, VEGF appears to play a principle role in many pathological states and processes related to neovascularization. Regulation of VEGF expression in tissues affected by the various conditions described above could therefore be key in treatment or preventative therapies associated with hypoxia.

The term “isolated” when used to describe the various polypeptides described herein, means polypeptides that have been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would

typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the TIE ligand's natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes nucleic acid molecules contained in cells that ordinarily express an TIE ligand of the present invention, where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native amino acid sequence.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule. Deletional variants include those having C- and/or N-terminal deletions

(truncations) as well as variants with internal deletions of one or more amino acids. The preferred deletional variants of the present invention contain deletions outside the fibrinogen-like domain of a native TIE ligand of the present invention.

The amino acid sequence variants of the present invention may contain various combinations of amino acid substitutions, insertions and/or deletions, to produce molecules with optimal characteristics.

The amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately.

I. Charged Amino Acids

Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine, glutamine

Aliphatic Residues: glycine, alanine, valine, leucine, isoleucine

Non-polar Residues: cysteine, methionine, proline

Aromatic Residues: phenylalanine, tyrosine, tryptophan

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another. Variants obtained by non-conservative substitutions are expected to result in significant changes in the biological properties/function of the obtained variant

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions may be introduced into regions not directly involved in the interaction with a native TIE receptor. Deletions are preferably performed outside the fibrinogen-like regions at the C-terminus of the TIE ligands of the present invention.

Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence

5 insertions of single or multiple amino acid residues. Intrasequence insertions (i.e. insertions within the TIE ligand amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5 residues, more preferably 1 to 3 residues. Examples of terminal insertions include the TIE ligands with an N-terminal methionyl residue, an artifact of its direct expression in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the TIE ligand molecule to facilitate the secretion of the mature TIE ligand from recombinant host cells. Such signal sequences will generally be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include, for example, STII or Ipp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertional variants of the native TIE ligand molecules include the fusion of the N- or C-terminus of the TIE ligand molecule to immunogenic polypeptides, e.g. bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin regions (preferably immunoglobulin constant regions), albumin, or ferritin, as described in WO 89/02922 published on 6 April 1989.

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15 Since it is often difficult to predict in advance the characteristics of a variant TIE ligand, it will be appreciated that some screening will be needed to select the optimum variant.

Amino acid sequence variants of native TIE ligands of the present invention are prepared by methods known in the art by introducing appropriate nucleotide changes into a native or variant TIE ligand DNA, or by *in vitro* synthesis of the desired polypeptide. There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. With the exception of naturally-occurring alleles, which do not require the manipulation of the DNA sequence encoding the TIE ligand, the amino acid sequence variants of TIE are preferably constructed by mutating the DNA, either to arrive at an allele or an amino acid sequence variant that does not occur in nature.

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25 One group of the mutations will be created within the domain or domains of the TIE ligands of the present invention identified as being involved in the interaction with a TIE receptor, e.g. TIE-1 or TIE-2.

Alternatively or in addition, amino acid alterations can be made at sites that differ in TIE ligands from various species, or in highly conserved regions, depending on the goal to be achieved.

Sites at such locations will typically be modified in series, e.g. by (1) substituting first with conservative choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue or residues, or (3) inserting residues of the same or different class adjacent to the located site, or combinations of options 1-3.

One helpful technique is called "alanine scanning" (Cunningham and Wells, Science 244, 1081-1085 [1989]). Here, a residue or group of target residues is identified and substituted by alanine or polyalanine. Those domains demonstrating functional sensitivity to the alanine substitutions are then refined by introducing further or other substituents at or for the sites of alanine substitution.

After identifying the desired mutation(s), the gene encoding an amino acid sequence variant of a TIE ligand can, for example, be obtained by chemical synthesis as hereinabove described.

More preferably, DNA encoding a TIE ligand amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the ligand. Site-directed (site-specific) mutagenesis allows the production of ligand variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are well known in the art, as exemplified by publications such as, Edelman *et al.*, DNA 2, 183 (1983). As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, Third Cleveland Symposium on Macromolecules and Recombinant DNA, A. Walton, ed., Elsevier, Amsterdam (1981). This and other phage vectors are commercially available and their use is well known to those skilled in the art. A versatile and

efficient procedure for the construction of oligodeoxyribonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller, M.J. and Smith, M., Nucleic Acids Res. 10, 6487-6500 [1982]). Also, plasmid vectors that contain a single-stranded phage origin of replication (Veira *et al.*, Meth. Enzymol. 153, 3 [1987]) may be employed to obtain single-stranded DNA. Alternatively, nucleotide substitutions are introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al.*, Proc. Natl. Acad. Sci. USA 75, 5765 (1978). This primer is then annealed with the single-stranded protein sequence-containing vector, and subjected to DNA-polymerizing enzymes such as, *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells such as JP101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region may be removed and placed in an appropriate expression vector for protein production.

The PCR technique may also be used in creating amino acid sequence variants of a TIE ligand. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results

in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more) part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 µg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp^R kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 µl. The reaction mixture is overlaid with 35 µl mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly on ice, and then 1 µl Thermus aquaticus (Taq) DNA polymerase (5 units/ µl), purchased from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows:

2 min. 55°C,

30 sec. 72°C, then 19 cycles of the following:

30 sec. 94°C,

30 sec. 55°C, and

30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50 vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.* [Gene 34, 315 (1985)]. The starting material is the plasmid (or vector) comprising the TIE ligand DNA to be mutated. The codon(s) within the TIE ligand to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the DNA encoding the TIE ligand. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction site but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated TIE ligand DNA sequence.

Additionally, the so-called phagemid display method may be useful in making amino acid sequence variants of native or variant TIE ligands. This method involves (a) constructing a replicable expression vector comprising a first gene encoding an receptor to be mutated, a second gene encoding at least a portion of a natural or wild-type phage coat protein wherein the first and second genes are heterologous, and a transcription regulatory element operably linked to the first and second genes, thereby forming a gene fusion encoding a fusion protein; (b) mutating the vector at one or more selected positions within the first gene thereby forming a family of related plasmids; (c) transforming suitable host cells with the plasmids; (d) infecting the transformed host cells with a helper phage having a gene encoding the phage coat protein; (e) culturing the transformed infected host cells under conditions suitable for forming recombinant phagemid particles containing at least a portion of the plasmid and capable of transforming the host, the conditions adjusted so that no more than a minor amount of phagemid particles display more than one copy of the fusion protein on the surface of the particle; (f) contacting the phagemid particles with a suitable antigen so that at least a portion of the phagemid particles bind to the antigen; and (g) separating the phagemid

particles that bind from those that do not. Steps (d) through (g) can be repeated one or more times. Preferably in this method the plasmid is under tight control of the transcription regulatory element, and the culturing conditions are adjusted so that the amount or number of phagemid particles displaying more than one copy of the fusion protein on the surface of the particle is less than about 1%. Also, preferably, the amount of phagemid particles displaying more than one copy of the fusion protein is less than 10% of the amount of phagemid particles displaying a single copy of the fusion protein. Most preferably, the amount is less than 20%. Typically in this method, the expression vector will further contain a secretory signal sequence fused to the DNA encoding each subunit of the polypeptide and the transcription regulatory element will be a promoter system. Preferred promoter systems are selected from lac Z, λ_{PL} , tac, T7 polymerase, tryptophan, and alkaline phosphatase promoters and combinations thereof. Also, normally the method will employ a helper phage selected from M13K07, M13R408, M13-VCS, and Phi X 174. The preferred helper phage is M13K07, and the preferred coat protein is the M13 Phage gene III coat protein. The preferred host is *E. coli*, and protease-deficient strains of *E. coli*.

Further details of the foregoing and similar mutagenesis techniques are found in general textbooks, such as, for example, Sambrook *et al.*, Molecular Cloning: A laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), and Current Protocols in Molecular Biology, Ausubel *et al.*, eds., Wiley-Interscience, 1991.

“Immunoadhesins” are chimeras which are traditionally constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins). Such structures are well known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor* [Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA 84, 2936-2940 (1987)]; CD4* [Capon *et al.*, Nature 337, 525-531 (1989); Traunecker *et al.*, Nature 339, 68-70 (1989); Zettmeissl *et al.*, DNA Cell Biol. USA 9, 347-353 (1990); Byrn *et al.*, Nature 344, 667-670 (1990)]; L-selectin (homing receptor) [Watson *et al.*, J. Cell. Biol. 110, 2221-2229 (1990); Watson *et al.*, Nature 349, 164-167 (1991)]; CD44* [Aruffo *et al.*, Cell 61, 1303-1313 (1990)]; CD28* and B7* [Linsley *et al.*, J. Exp. Med. 173, 721-730 (1991)]; CTLA-4* [Lisley *et al.*, J. Exp. Med. 174, 561-569 (1991)]; CD22* [Stamenkovic *et al.*, Cell 66, 1133-1144 (1991)]; TNF receptor [Ashkenazi *et al.*, Proc. Natl.

Acad. Sci. USA 88, 10535-10539 (1991); Lesslauer *et al.*, *Eur. J. Immunol.* 27, 2883-2886 (1991); Peppel *et al.*, *J. Exp. Med.* 174, 1483-1489 (1991)]; NP receptors [Bennett *et al.*, *J. Biol. Chem.* 266, 23060-23067 (1991)]; IgE receptor α -chain* [Ridgway and Gorman, *J. Cell. Biol.* 115, abstr. 1448 (1991)]; HGF receptor [Mark, M.R. *et al.*, 1992, *J. Biol. Chem.* submitted], where the asterisk (*) indicates that the receptor is member of the immunoglobulin superfamily.

Ligand-immunoglobulin chimeras are also known, and are disclosed, for example, in U.S. Patents Nos. 5,304,640 (for L-selectin ligands); 5,316,921 and 5,328,837 (for HGF variants). These chimeras can be made in a similar way to the construction of receptor-immunoglobulin chimeras.

Covalent modifications of the TIE ligands of the present invention are included within the scope herein. Such modifications are traditionally introduced by reacting targeted amino acid residues of the TIE ligand with an organic derivatizing agent that is capable of reacting with selected sides or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-TIE ligand antibodies for immunoaffinity purification of the recombinant. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Cysteinyll residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyll residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazolyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

5 Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

10 Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

15 The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

20 Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($\text{R}'\text{-N}=\text{C}=\text{N-R}'$) such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

25 Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α -amino groups of lysine, arginine,

and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The molecules may further be covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S.S.N. 07/275,296 or U.S. patents 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Derivatization with bifunctional agents is useful for preparing intramolecular aggregates of the TIE ligand with polypeptides as well as for cross-linking the TIE ligand polypeptide to a water insoluble support matrix or surface for use in assays or affinity purification. In addition, a study of interchain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional maleimides. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. Patent Nos. 3,959,642; 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed for protein immobilization and cross-linking.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and aspariginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

Other derivatives comprise the novel peptides of this invention covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such as polyethylene glycol, polypropylene glycol.

The TIE ligands may be linked to various nonproteinaceous polymers, such as polyethylene glycol (PEG), polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. These variants, just as the immunoadhesins of the present invention are expected to have longer half-lives than the corresponding native TIE ligands.

The TIE ligands may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980).

The term "native TIE receptor" is used herein to refer to a TIE receptor of any animal species, including, but not limited to, humans, other higher primates, e.g. monkeys, and rodents, e.g. rats and mice. The definition specifically includes the TIE-2 receptor, disclosed, for example, in PCT Application Serial No. WO 95/13387 (published 18 May 1995), and the endothelial cell receptor tyrosine kinase termed "TIE" in PCT Application Publication No. WO 93/14124 (published 22 July 1993), and preferably is TIE-2.

B. ANTI-TIE LIGAND ANTIBODIES

The present invention covers agonist and antagonist antibodies, specifically binding the TIE ligands. The antibodies may be monoclonal or polyclonal, and include, without limitation, mature

antibodies, antibody fragments (e.g. Fab, F(ab')₂, Fv, etc.), single-chain antibodies and various chain combinations.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) specifically binding a TIE ligand of the present invention and antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-TIE ligand antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

Polyclonal antibodies to a TIE ligand of the present invention generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the TIE ligand and an adjuvant. It may be useful to conjugate the TIE ligand or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, *e.g.* keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glytaraldehyde, succinic anhydride, SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.

Animals are immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 µg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freud's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freud's complete adjuvant by

subcutaneous injection at multiple sites. 7 to 14 days later the animals are bled and the serum is assayed for anti-TIE ligand antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal boosted with the conjugate of the same TIE ligand, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the anti-TIE ligand monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, *Nature* 256:495 (1975), or may be made by recombinant DNA methods [Cabilly, *et al.*, U.S. Pat. No. 4,816,567].

In the hybridoma method, a mouse or other appropriate host animal, such as hamster is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)].

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT

medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol. 133:3001 (1984); Brodeur, *et al.*, Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987)].

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the TIE ligand. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson & Pollard, Anal. Biochem. 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed

into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison, *et al.*, Proc. Nat. Acad. Sci. 81, 6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-TIE ligand monoclonal antibody herein.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a TIE ligand of the present invention and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; biotin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter, *et al.*, Nature 144:945 (1962);

David, *et al.*, Biochemistry 13:1014 (1974); Pain, *et al.*, J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays.

5 Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be a TIE ligand or an immunologically reactive portion thereof) to compete with the test sample analyte (TIE ligand) for binding with a limited amount of antibody. The amount of TIE ligand in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To

10 facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample

15 analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA

20 assay, in which case the detectable moiety is an enzyme.

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed

25 following the method of Winter and co-workers [Jones *et al.*, Nature 321, 522-525 (1986); Riechmann *et al.*, Nature 332, 323-327 (1988); Verhoeven *et al.*, Science 239, 1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly, supra),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

5 It is important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to
10 those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e. the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from
15 the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding. For further details see U.S. application Serial No. 07/934,373 filed 21 August 1992, which is a continuation-in-part of application Serial No. 07/715,272 filed 14 June 1991.

20 Alternatively, it is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line
25 immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g. Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA **90**, 2551-255 (1993); Jakobovits *et al.*, Nature **362**, 255-258 (1993).

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a particular TIE ligand, the other one is for any other antigen, and preferably for another ligand. For example, bispecific antibodies specifically binding two different TIE ligands are within the scope of the present invention.

Methods for making bispecific antibodies are known in the art.

Traditionally, the recombinant production of bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Millstein and Cuello, Nature **305**, 537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in PCT application publication No. WO 93/08829 (published 13 May 1993), and in Traunecker *et al.*, EMBO **10**, 3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, and second and third constant regions of an immunoglobulin heavy chain (CH2 and CH3). It is preferred to have the first heavy chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no

particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in copending application Serial No. 07/931,811 filed 17 August 1992.

For further details of generating bispecific antibodies see, for example, Suresh *et al.*, Methods in Enzymology 121, 210 (1986).

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (PCT application publication Nos. WO 91/00360 and WO 92/200373; EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

The term "agonist" is used to refer to peptide and non-peptide analogs of the native TIE ligands of the present invention and to antibodies specifically binding such native TIE ligands, provided that they have the ability to signal through a native TIE receptor (e.g. TIE-2). In other words, the term "agonist" is defined in the context of the biological role of the TIE receptor, and not in relation to the biological role of a native TIE ligand, which, as noted before, may be an agonist or antagonist of the TIE receptor biological function. Preferred agonists are promoters of vascularization.

The term "antagonist" is used to refer to peptide and non-peptide analogs of the native TIE ligands of the present invention and to antibodies specifically binding such native TIE ligands, provided that they have the ability to inhibit the biological function of a native TIE receptor (e.g. TIE-2). Again, the term "antagonist" is defined in the context of the biological role of the TIE

receptor, and not in relation to the biological activity of a native TIE ligand, which may be either an agonist or an antagonist of the TIE receptor biological function. Preferred antagonists are inhibitors of vasculogenesis.

5 C. CLONING AND EXPRESSION OF THE TIE LIGANDS

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included.

10 The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

15 Expression and cloning vectors are well known in the art and contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. The selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the TIE ligand molecule that is inserted into the vector. If the signal sequence is heterologous, it should be selected such that it is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell.

Heterologous signal sequences suitable for prokaryotic host cells are preferably prokaryotic signal sequences, such as the α -amylase, ompA, ompC, ompE, ompF, alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the yeast invertase, amylase, alpha factor, or acid phosphatase leaders may, for example, be used. In mammalian cell expression mammalian signal sequences are most suitable. The listed signal sequences are for illustration only, and do not limit the scope of the present invention in any way.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enabled the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomes, and includes origins of replication or autonomously replicating sequences. Such sequence are well known for a variety of bacteria, yeast and viruses. The origin of replication from the well-known plasmid pBR322 is suitable for most gram negative bacteria, the 2 μ plasmid origin for yeast and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Origins of replication are not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter). Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA is also cloned by insertion into the host genome. This is readily accomplished using Bacillus species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in Bacillus genomic DNA. Transfection of Bacillus with this

vector results in homologous recombination with the genome and insertion of the DNA encoding the desired heterologous polypeptide. However, the recovery of genomic DNA is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the encoded polypeptide molecule.

5 (iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This is a gene that encodes a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts. Typical
10 selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells
15 that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern *et al.*, J. Molec. Appl. Genet. 1, 327 (1982)], mycophenolic acid [Mulligan *et al.*, Science 209, 1422 (1980)], or hygromycin [Sudgen *et al.*, Mol. Cel.. Biol. 5, 410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to
20 convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Other examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR) or thymidine kinase. Such markers enable the identification of cells which were competent to take up the desired nucleic acid. The mammalian cell transformants are placed under
25 selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the desired polypeptide.

Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the desired polypeptide are synthesized from the amplified DNA.

5 For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium which lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Nat'l. Acad. Sci. USA 77, 4216 (1980). A particularly useful DHFR is a mutant DHFR that is highly resistant to MTX (EP 10 117,060). This selection agent can be used with any otherwise suitable host, e.g. ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR. The DNA encoding DHFR and the desired polypeptide, respectively, then is amplified by exposure to an agent (methotrexate, or MTX) that inactivates the DHFR. One ensures that the cell requires more DHFR (and consequently amplifies all exogenous DNA) by selecting only for cells that can grow in successive rounds of ever- 15 greater MTX concentration. Alternatively, hosts co-transformed with genes encoding the desired polypeptide, wild-type DHFR, and another selectable marker such as the neo gene can be identified using a selection agent for the selectable marker such as G418 and then selected and amplified using methotrexate in a wild-type host that contains endogenous DHFR. (See also U.S. Patent No. 4,965,199).

20 A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, 1979, Nature 282:39; Kingsman *et al.*, 1979, Gene 7:141; or Tschemper *et al.*, 1980, Gene 10:157). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, 1977, Genetics 85:12). The presence of the trp1 lesion in the yeast host cell genome then provides an effective 25 environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2 deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

(iv) Promoter Component

Expression vectors, unlike cloning vectors, should contain a promoter which is recognized by the host organism and is operably linked to the nucleic acid encoding the desired polypeptide. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the desired polypeptide by removing them from their gene of origin by restriction enzyme digestion, followed by insertion 5' to the start codon for the polypeptide to be expressed. This is not to say that the genomic promoter for a TIE ligand is not usable. However, heterologous promoters generally will result in greater transcription and higher yields of expressed TIE ligands as compared to the native TIE ligand promoters.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang *et al.*, Nature 275:615 (1978); and Goeddel *et al.*, Nature 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res. 8:4057 (1980) and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the tac promoter (H. de Boer *et al.*, Proc. Nat'l. Acad. Sci. USA 80:21-25 (1983)). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding a TIE ligand (Siebenlist *et al.*, Cell 20:269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding a TIE ligand.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.* J. Biol. Chem. 255:2073 (1980)) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg. 7:149 (1978); and Holland, Biochemistry 17:4900

(1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytocrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

TIE ligand transcription from vectors in mammalian host cells may be controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat shock promoters, and from the promoter normally associated with the TIE ligand sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication [Fiers *et al.*, Nature 273:113 (1978), Mulligan and Berg, Science 209, 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA 78, 7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway *et al.*, Gene

18, 355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in US 4,419,446. A modification of this system is described in US 4,601,978. See also, Gray *et al.*, Nature 295, 503-508 (1982) on expressing cDNA encoding human immune interferon in monkey cells; Reyes *et al.*, Nature 297, 598-601 (1982) on expressing human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79, 5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, Proc. Natl. Acad. Sci., USA 79, 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse HIN-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the TIE ligands of the present invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' [Laimins *et al.*, Proc. Natl. Acad. Sci. USA 78, 993 (1981)] and 3' [Lasky *et al.*, Mol. Cell. Biol. 3, 1108 (1983)] to the transcription unit, within an intron [Banerji *et al.*, Cell 33, 729 (1983)] as well as within the coding sequence itself [Osborne *et al.*, Mol. Cell. Biol. 4, 1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297, 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the TIE ligand DNA, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the

5 termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the TIE ligand. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components, the desired coding and control sequences, employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

10 For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res. 9, 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology 65, 499 (1980).

15 Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding a TIE ligand. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by clones DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of a TIE ligand.

25 Other methods, vectors, and host cells suitable for adaptation to the synthesis of the TIE polypeptides in recombinant vertebrate cell culture are described in Getting *et al.*, Nature 293, 620-625 (1981); Mantel *et al.*, Nature 281, 40-46 (1979); Levinson *et al.*; EP 117,060 and EP 117,058.

A particularly useful plasmid for mammalian cell culture expression of the TIE ligand polypeptides is pRK5 (EP 307,247), along with its derivatives, such as, pRK5D that has an sp6 transcription initiation site followed by an SfiI restriction enzyme site preceding the Xho/NotII cDNA cloning sites, and pRK5B, a precursor of pRK5D that does not contain the SfiI site; see, Holmes et al., Science 253, 1278-1280 (1991).

(vii) Construction and analysis of vectors

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequences by the methods of Messing *et al.*, Nuclei Acids Res. 9, 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology 65, 499 (1980).

(viii) Transient expression vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding a TIE ligand. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high level of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, supra, pp. 16.17-16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive screening of such polypeptides for desired biological or physiological properties. Thus transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of native TIE ligands with the requisite biological activity.

(ix) Suitable exemplary vertebrate cell vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of a TIE ligand (including functional derivatives of native proteins) in recombinant vertebrate cell culture are described in Gething *et al.*, Nature 293, 620-625 (1981); Mantei *et al.*, Nature 281, 40-46 (1979);
 5 Levinson *et al.*, EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of a TIE ligand is pRK5 (EP 307,247) or pSVI6B (PCT Publication No. WO 91/08291).

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast or higher eukaryote cells described above. Suitable prokaryotes include gram negative or gram positive
 10 organisms, for example E. coli or bacilli. A preferred cloning host is E. coli 294 (ATCC 31,446) although other gram negative or gram positive prokaryotes such as E. coli B, E. coli X1776 (ATCC 31,537), E. coli W3110 (ATCC 27,325), Pseudomonas species, or Serratia Marcesans are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors herein. Saccharomyces cerevisiae, or common baker's yeast, is the most
 15 commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species and strains are commonly available and useful herein, such as S. pombe [Beach and Nurse, Nature 290, 140 (1981)], Kluyveromyces lactis [Louvencourt *et al.*, J. Bacteriol. 737 (1983)]; Yarrowia (EP 402,226); Pichia pastoris (EP 183,070), Trichoderma reesia (EP 244,234), Neurospora crassa [Case *et al.*, Proc. Natl. Acad. Sci. USA 76, 5259-5263 (1979)]; and Aspergillus hosts such
 20 as A. nidulans [Ballance *et al.*, Biochem. Biophys. Res. Commun. 112, 284-289 (1983); Tilburn *et al.*, Gene 26, 205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA 81, 1470-1474 (1984)] and A. niger [Kelly and Hynes, EMBO J. 4, 475-479 (1985)].

Suitable host cells may also derive from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture
 25 is workable, whether from vertebrate or invertebrate culture, although cells from mammals such as humans are preferred. Examples of invertebrate cells include plants and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito),

Drosophila melangaster (fruitfly), and Bombyx mori host cells have been identified. See, e.g. Luckow *et al.*, Bio/Technology 6, 47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature 315, 592-594 (1985). A variety of such viral strains are publicly available, e.g. the L-1 variant of Autographa californica NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells.

Generally, plant cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain the TIE ligand DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding a TIE ligand is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the TIE ligand DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen. 1, 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) is per se well known. See Tissue Culture, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cell line [293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol. 36, 59 (1977)]; baby hamster kidney cells 9BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR [CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77, 4216 (1980)]; mouse sertolli cells [TM4, Mather, Biol. Reprod. 23, 243-251 (1980)]; monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells [Mather *et al.*, Annals N.Y. Acad. Sci.

383, 44068 (1982)]; MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Particularly preferred host cells for the purpose of the present invention are vertebrate cells producing the TIE ligands of the present invention.

5 Host cells are transfected and preferably transformed with the above-described expression or cloning vectors and cultured in conventional nutrient media modified as is appropriate for inducing promoters or selecting transformants containing amplified genes.

Prokaryotes cells used to produced the TIE ligands of this invention are cultured in suitable media as describe generally in Sambrook *et al.*, supra.

10 Mammalian cells can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enzymol. 58, 44 (1979); Barnes and Sato, Anal. Biochem. 102, 255 (1980), US 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 15 90/03430; WO 87/00195 or US Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug) trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH and the like, suitably are those previously used with the host cell selected for cloning or expression, as the case may be, and will be apparent to the ordinary artisan.

20 25 The host cells referred to in this disclosure encompass cells in *in vitro* cell culture as well as cells that are within a host animal or plant.

It is further envisioned that the TIE ligands of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the particular TIE ligand.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA 77, 5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as a site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to the surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hse *et al.*, Am. J. Clin. Pharm. 75, 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any animal. Conveniently, the antibodies may be prepared against a native TIE ligand polypeptide of the present invention, or against a synthetic peptide based on the DNA sequence provided herein as described further hereinbelow.

The TIE ligand may be produced in host cells in the form of inclusion bodies or secreted into the periplasmic space or the culture medium, and is typically recovered from host cell lysates. The recombinant ligands may be purified by any technique allowing for the subsequent formation of a stable protein.

5 When the TIE ligand is expressed in a recombinant cell other than one of human origin, it is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the TIE ligand from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as to the ligand. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then
10 separated. The TIE ligand may then be purified from the soluble protein fraction. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.
15

Functional derivatives of the TIE ligands in which residues have been deleted, inserted and/or substituted are recovered in the same fashion as the native ligands, taking into account of any substantial changes in properties occasioned by the alteration. For example, fusion of the TIE ligand with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to absorb the fusion.
20 Immunoaffinity columns such as a rabbit polyclonal anti-TIE ligand column can be employed to absorb TIE ligand variants by binding to at least one remaining immune epitope. A protease inhibitor, such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of
25 adventitious contaminants. The TIE ligands of the present invention are conveniently purified by affinity chromatography, based upon their ability to bind to a TIE receptor, e.g. TIE-2.

One skilled in the art will appreciate that purification methods suitable for native TIE ligands may require modification to account for changes in the character of a native TIE ligand or its variants upon expression in recombinant cell culture

D. USE OF THE TIE LIGANDS, NUCLEIC ACID MOLECULES AND ANTIBODIES

The TIE ligands of the present invention are useful in promoting the survival and/or growth and/or differentiation of TIE receptor (e.g. TIE-2 receptor) expressing cells in cell culture.

The TIE ligands may be additionally used to identify cells which express native TIE receptors, e.g. the TIE-2 receptor. To this end, a detectably labeled ligand is contacted with a target cell under condition permitting its binding to the TIE receptor, and the binding is monitored.

The TIE ligands herein may also be used to identify molecules exhibiting a biological activity of a TIE ligand, for example, by exposing a cell expressing a TIE ligand herein to a test molecule, and detecting the specific binding of the test molecule to a TIE (e.g. TIE-2) receptor, either by direct detection, or base upon secondary biological effects. This approach is particularly suitable for identifying new members of the TIE ligand family, or for screening peptide or non-peptide small molecule libraries.

The TIE ligands disclosed herein are also useful in screening assays designed to identify agonists or antagonists of a native TIE (e.g. TIE-2) receptor that play an important role in bone development, maturation or growth, or in muscle growth or development and/or promote or inhibit angiogenesis. For example, antagonists of the TIE-2 receptor may be identified based upon their ability to block the binding of a TIE ligand of the present invention to a native TIE receptor, as measured, for example, by using BiAcore biosensor technology (BIAcore; Pharmacia Biosensor, Midscataway, N.J.); or by monitoring their ability to block the biological response caused by a biologically active TIE ligand herein. Biological responses that may be monitored include, for example, the phosphorylation of the TIE-2 receptor or downstream components of the TIE-2 signal transduction pathway, or survival, growth or differentiation of cells expressing the TIE-2 receptor. Cell-based assays, utilizing cells that do not normally the TIE-2 receptor, engineered to express this

receptor, or to coexpress the TIE-2 receptor and a TIE ligand of the present invention, are particularly convenient to use.

In a particular embodiment, small molecule agonists and antagonists of a native TIE receptor, e.g. the TIE-2 receptor, can be identified, based upon their ability to interfere with the TIE ligand/TIE receptor interaction. There are numerous ways for measuring the specific binding of a test molecule to a TIE receptor, including, but not limited to detecting or measuring the amount of a test molecule bound to the surface of intact cells expressing the TIE receptor, cross-linked to the TIE receptor in cell lysates, or bound to the TIE receptor *in vitro*.

Detectably labeled TIE ligands include, for example, TIE ligands covalently or non-covalently linked to a radioactive substances, e.g. ^{125}I , a fluorescent substance, a substance having enzymatic activity (preferably suitable for colorimetric detection), a substrate for an enzyme (preferably suitable for colorimetric detection), or a substance that can be recognized by a(n) (detectably labeled) antibody molecule.

The assays of the present invention may be performed in a manner similar to that described in PCT Publication WO 96/11269, published 18 April 1996.

The TIE ligands of the present invention are also useful for purifying TIE receptors, e.g. TIE-2 receptors, optionally used in the form of immunoadhesins, in which the TIE ligand or the TIE receptor binding portion thereof is fused to an immunoglobulin heavy or light chain constant region.

The nucleic acid molecules of the present invention are useful for detecting the expression of TIE ligands in cells or tissue sections. Cells or tissue sections may be contacted with a detectably labeled nucleic acid molecule encoding a TIE ligand of the present invention under hybridizing conditions, and the presence of mRNA hybridized to the nucleic acid molecule determined, thereby detecting the expression of the TIE ligand.

Antibodies of the present invention may, for example, be used in immunoassays to measure the amount of a TIE ligand in a biological sample. The biological sample is contacted with an antibody or antibody mixture specifically binding the a TIE ligand of the present invention, and the amount of the complex formed with a ligand present in the test sample is measured.

Antibodies to the TIE ligands herein may additionally be used for the delivery of cytotoxic molecules, e.g. radioisotopes or toxins, or therapeutic agents to cells expressing a corresponding TIE receptor. The therapeutic agents may, for example, be other TIE ligands, including the TIE-2 ligand, members of the vascular endothelial growth factor (VEGF) family, or known anti-tumor agents, and agents known to be associated with muscle growth or development, or bone development, maturation, or growth.

Anti-TIE ligand antibodies are also suitable as diagnostic agents, to detect disease states associated with the expression of a TIE (e.g. TIE-2) receptor. Thus, detectably labeled TIE ligands and antibody agonists of a TIE receptor can be used for imaging the presence of angiogenesis.

In addition, the new TIE ligands herein can be used to promote neovascularization, and may be useful for inhibiting tumor growth.

Further potential therapeutic uses include the modulation of muscle and bone development, maturation, or growth.

For therapeutic use, the TIE ligands or anti-TIE ligand antibodies of the present invention are formulated as therapeutic composition comprising the active ingredient(s) in admixture with a pharmacologically acceptable vehicle, suitable for systemic or topical application. The pharmaceutical compositions of the present invention are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or PEG.

5 The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

10 Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

15 The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional routes, topical administration, or by sustained release systems.

20 Suitable examples of sustained release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Patent 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman *et al.*, 1983, "Biopolymers" 22 (1): 547-556), poly (2-hydroxyethyl-methacrylate) (R. Langer, *et al.*, 1981, "J. Biomed. Mater. Res." 15: 167-277 and R. Langer, 1982, Chem. Tech." 12: 98-105), ethylene vinyl acetate (R. Langer *et al.*, Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988A). Sustained release compositions also include liposomes. Liposomes containing a molecule within the scope of the present invention are prepared by methods known *per se*: DE 3,218,121A; Epstein *et al.*, 1985, "Proc. Natl. Acad. Sci. USA" 82: 3688-3692; 25 Hwang *et al.*, 1980, "Proc. Natl. Acad. Sci. USA" 77: 4030-4034; EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142641A; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324A. Ordinarily the liposomes are of the small (about 200-800

Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal NT-4 therapy.

An effective amount of a molecule of the present invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assays.

Further details of the invention will be apparent from the following non-limiting examples.

Reference Example 1

Identification of the FLS 139 ligand

FLS139 was identified in a cDNA library prepared from human fetal liver mRNA obtained from Clontech Laboratories, Inc. Palo Alto, CA USA, catalog no. 64018-1, following the protocol described in "Instruction Manual: Superscript® Lambda System for cDNA Synthesis and λ cloning," cat. No. 19643-014, Life Technologies, Gaithersburg, MD, USA which is herein incorporated by reference. Unless otherwise noted, all reagents were also obtained from Life Technologies. The overall procedure can be summarized into the following steps: (1) First strand synthesis; (2) Second strand synthesis; (3) Adaptor addition; (4) Enzymatic digestion; (5) Gel isolation of cDNA; (6) Ligation into vector; and (7) Transformation.

First strand synthesis:

Not1 primer-adaptor (Life Tech., 2 µl, 0.5 µg/µl) was added to a sterile 1.5 ml microcentrifuge tube to which was added poly A+ mRNA (7µl, 5µg). The reaction tube was heated to 70° C for 5 minutes or time sufficient to denature the secondary structure of the mRNA. The

reaction was then chilled on ice and 5X First strand buffer (Life Tech., 4 μ l), 0.1 M DTT (2 μ l) and 10 mM dNTP Mix (Life Tech., 1 μ l) were added and then heated to 37°C for 2 minutes to equilibrate the temperature. Superscript II® reverse transcriptase (Life Tech., 5 μ l) was then added, the reaction tube mixed well and incubated at 37°C for 1 hour, and terminated by placement on ice.

5 The final concentration of the reactants was the following: 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM MgCl₂; 10 mM DTT; 500 μ M each dATP, dCTP, dGTP and dTTP; 50 μ g/ml Not 1 primer-adapter; 5 μ g (250 μ g/ml) mRNA; 50,000 U/ml Superscript II® reverse transcriptase.

Second strand synthesis:

10 While on ice, the following reagents were added to the reaction tube from the first strand synthesis, the reaction well mixed and allowed to react at 16°C for 2 hours, taking care not to allow the temperature to go above 16°C: distilled water (93 μ l); 5X Second strand buffer (30 μ l); dNTP mix (3 μ l); 10 U/ μ l E. Coli DNA ligase (1 μ l); 10 U/ μ l E. Coli DNA polymerase I (4 μ l); 2 U/ μ l E. Coli RNase H (1 μ l). 10 U T4 DNA Polymerase (2 μ l) was added and the reaction continued to incubate at 16°C for another 5 minutes. The final concentration of the reaction was the following:

15 25 mM Tris-HCl (pH 7.5); 100 mM KCl; 5 mM MgCl₂; 10 mM (NH₄)₂SO₄; 0.15 mM β -NAD⁺; 250 μ M each dATP, dCTP, dGTP, dTTP; 1.2 mM DTT; 65 U/ml DNA ligase; 250 U/ml DNA polymerase I; 13 U/ml Rnase H. The reaction has halted by placement on ice and by addition of 0.5 M EDTA (10 μ l), then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 150 μ l). The aqueous phase was removed, collected and diluted into 5M NaCl (15 μ l) and absolute ethanol (-20°C, 400 μ l) and centrifuged for 2 minutes at 14,000 x g. The supernatant was carefully removed from the resulting DNA pellet, the pellet resuspended in 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was again removed and the pellet dried in a speedvac.

Adapter addition

25 The following reagents were added to the cDNA pellet from the Second strand synthesis above, and the reaction was gently mixed and incubated at 16°C for 16 hours: distilled water (25 μ l); 5X T4 DNA ligase buffer (10 μ l); Sal I adapters (10 μ l); T4 DNA ligase (5 μ l). The final

composition of the reaction was the following: 50 mM Tris-HCl (pH 7.6); 10 mM MgCl₂; 1 mM ATP; 5% (w/v) PEG 8000; 1 mM DTT; 200 µg/ml Sal 1 adapters; 100 U/ml T4 DNA ligase. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 µl), the aqueous phase removed, collected and diluted into 5M NaCl (8 µl) and absolute ethanol (-20°C, 250 µl). This was then centrifuged for 20 minutes at 14,000 x g, the supernatant removed and the pellet was resuspended in 0.5 ml 70% ethanol, and centrifuged again for 2 minutes at 14,000 x g. Subsequently, the supernatant was removed and the resulting pellet dried in a speedvac and carried on into the next procedure.

Enzymatic digestion:

To the cDNA prepared with the Sal 1 adapter from the previous paragraph was added the following reagents and the mixture was incubated at 37°C for 2 hours: DEPC-treated water (41 µl); Not 1 restriction buffer (REACT, Life Tech., 5 µl), Not 1 (4 µl). The final composition of this reaction was the following: 50 mM Tris-HCl (pH 8.0); 10 mM MgCl₂; 100 mM NaCl; 1,200 U/ml Not 1.

Gel isolation of cDNA:

The cDNA is size fractionated by acrylamide gel electrophoresis on a 5% acrylamide gel, and any fragments which were larger than 1 Kb, as determined by comparison with a molecular weight marker, were excised from the gel. The cDNA was then electroeluted from the gel into 0.1 x TBE buffer (200 µl) and extracted with phenol:chloroform:isoamyl alcohol (25:24:1, 200 µl). The aqueous phase was removed, collected and centrifuged for 20 minutes at 14,000 x g. The supernatant was removed from the DNA pellet which was resuspended in 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was again discarded, the pellet dried in a speedvac and resuspended in distilled water (15 µl).

Ligation of cDNA into pRK5 vector:

The following reagents were added together and incubated at 16 °C for 16 hours: 5X T4 ligase buffer (3 µl); pRK5, Xho1, Not1 digested vector, 0.5 µg, 1 µl); cDNA prepared from previous paragraph (5 µl) and distilled water (6 µl). Subsequently, additional distilled water (70 µl) and 10 mg/ml tRNA (0.1 µl) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 µl) and absolute ethanol (-20° C, 250 µl). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 µl) for use in the subsequent procedure.

Transformation of library ligation into bacteria:

The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 µl). The bacteria vector mixture was then electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37° C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37° C) to allow the colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols. For example, Ausubel et al., 2.3.1.

Identification of FLS139

FLS139 can be identified in the human fetal liver library by any standard method known in the art, including the methods reported by Klein R.D. et al. (1996), *Proc. Natl. Acad. Sci.* 93, 7108-7113 and Jacobs (U.S. Patent No. 5,563,637 issued July 16, 1996). According to Klein et al. and Jacobs, cDNAs encoding novel secreted and membrane-bound mammalian proteins are identified by detecting their secretory leader sequences using the yeast invertase gene as a reporter system. The enzyme invertase catalyzes the breakdown of sucrose to glucose and fructose as well as the breakdown of raffinose to sucrose and melibiose. The secreted form of invertase is required for the

utilization of sucrose by yeast (*Saccharomyces cerevisiae*) so that yeast cells that are unable to produce secreted invertase grow poorly on media containing sucrose as the sole carbon and energy source. Both Klein R.D., *supra*, and Jacobs, *supra*, take advantage of the known ability of mammalian signal sequences to functionally replace the native signal sequence of yeast invertase.

5 A mammalian cDNA library is ligated to a DNA encoding a nonsecreted yeast invertase, the ligated DNA is isolated and transformed into yeast cells that do not contain an invertase gene. Recombinants containing the nonsecreted yeast invertase gene ligated to a mammalian signal sequence are identified based upon their ability to grow on a medium containing only sucrose or only raffinose as the carbon source. The mammalian signal sequences identified are then used to screen

10 a second, full-length cDNA library to isolate the full-length clones encoding the corresponding secreted proteins.

The nucleotide sequence of FLS139 is shown in Figure 1-A (SEQ. ID. NO: 16), while its amino acid sequence is shown in Figure 1-B (SEQ. ID. NO:17). FLS139 contains a fibrinogen-like domain exhibiting a high degree of sequence homology with the two known human ligands of the TIE-2 receptor (h-TIE2L1 and h-TIE2L2). Accordingly, FLS139 has been identified as a novel member of the TIE ligand family.

A clone of FLS139 was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, on _____ under the terms of the Budapest Treaty, and has been assigned the deposit number _____.

Example 1

Identification of NL1

NL1 was identified by screening the GenBank database using the computer program BLAST (Altshul et al., Methods in Enzymology 266:460-480 (1996)). The NL1 sequence shows homology

25 with known expressed sequence tag (EST) sequences T35448, T11442, and W77823. None of the known EST sequences have been identified as full length sequences, or described as ligands associated with the TIE receptors.

Following its identification, NL1 was cloned from a human fetal lung library prepared from mRNA purchased from Clontech, Inc. (Palo Alto, CA, USA), catalog # 6528-1, following the manufacturer's instructions. The library was screened by hybridization with synthetic oligonucleotide probes:

NL1.5-1 5'-GCTGACGAACCAAGGCAACTACAACTCCTGGT SEQ. ID. NO: 7

NL1.3-1 5'-TGCGGCCCGGACCAGTCCTCCATGGTCACCAGGAGTTTGTAG SEQ. ID. NO: 8

NL1.3-2 5'-GGTGGTGAAGTGGCTTGCCGTTGTGCCATGTAAA SEQ. ID. NO: 9

based on the ESTs found in the GenBank database. cDNA sequences were sequenced in their entirety.

The nucleotide and amino acid sequences of NL1 are shown in Figure 2 (SEQ. ID. NO:1) and Figure 3 (SEQ. ID. NO: 2), respectively.

NL1 shows a 23% sequence identity with both the TIE1 and the TIE2 ligand.

A clone of NL1 was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, on ____ under the terms of the Budapest Treaty, and has been assigned the deposit number ____.

Example 2

Identification of NL5 and NL8

An expressed sequence tag (EST) DNA database (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) was searched and ESTs were identified that showed homology to the FLS139 protein of Reference Example 1. To clone NL5 and NL8, a human fetal lung library prepared from mRNA purchased from Clontech, Inc. (Palo Alto, CA, USA), catalog # 6528-1 was used, following the manufacturer's instructions. The library was screened by hybridization with synthetic oligonucleotide probes:

NL5

NL5.5-1 5' CAGGTTATCCCAGAGATTTAATGCCACCA SEQ. ID. NO: 10

NL5.3-1 5' TTGGTGGGAGAAGTTGCCAGATCAGGTGGTGGCA SEQ. ID. NO: 11

NL5.3-2 5' TTCACACCATAACTGCATTGGTCCA SEQ. ID. NO: 12

5 NL8

NL8.5-1 5' ACGTAGTTCCAGTATGGTGTGAGCAGCAACTGGA SEQ. ID. NO: 13

NL8.3-1 5' AGTCCAGCCTCCACCCTCCAGTTGCT SEQ. ID. NO: 14

NL8.3-2 5' CCCCAGTCCTCCAGGAGAACCAGCA SEQ. ID. NO: 15

10 based on the ESTs found in the database. cDNA sequences were sequenced in their entireties. cDNA clones were sequenced. The entire nucleotide and deduced amino acid sequences of NL5 are shown in Figures 4 and 5 (SEQ. ID. Nos: 3 and 4). The entire nucleotide and deduced amino acid sequences of NL8 are shown in Figures 6 and 7 (SEQ. ID. Nos: 5 and 6).

15 Based on a BLAST and FastA sequence alignment analysis (using the ALIGN program) of the full-length sequences, NL5 shows a 24% sequence identity with both ligand 1 and ligand 2 of the TIE2 receptor. NL8 shows a 23% sequence identity with both ligand 1 and ligand 2 of the TIE2 receptor.

20 The fibrinogen domains of the TIE ligands NL1, NL5 and NL8 are 64-74 % identical. More specifically, the fibrinogen domain of NL1 is 74% identical with the fibrinogen domain of NL5 and 63% identical with the fibrinogen domain of NL8, while the fibrinogen domain of NL5 is 57% identical with the fibrinogen domain of NL8. Ligand 1 and ligand 2 of the TIE-2 receptor are 64% identical and 40-43% identical to NL1, NL5 and NL8.

25

Example 3**Northern Blot and *in situ* RNA Hybridization Analysis**

Expression of the NL1 and NL5 mRNA in human tissues was examined by Northern blot analysis. Human mRNA blots were hybridized to a ³²P-labeled DNA probe based on the full length cDNAs; the probes were generated by digesting and purifying the cDNA inserts. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5x SSPE; 2Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42° C. The blots were washed several times in 2x SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1x SSC; 0.1% SDS at 50° C. The blots were developed after overnight exposure by phosphorimager analysis (Fuji).

As shown in Figures 11 and 12, NL1 and NL5 mRNA transcripts were detected. Strong NL1 mRNA expression was detected in heart and skeletal muscle tissue and in the pancreas. NL5 mRNA was strongly expressed in skeletal muscle, and, to a lesser degree, heart, placenta and pancreas.

In situ hybridization results show that NL1 is expressed in the cartilage of developing long bones and in periosteum adjacent to differentiating osteoblasts. Expression was also observed in connective tissue at sites of synovial joint formation, in connective tissue septa, and in the periosteum of fetal body wall (Figures 8-A and 8-B).

In situ hybridization was performed using an optimized protocol, using PCR-generated ³³P-labeled riboprobes. (Lu and Gillett, Cell Vision 1: 169-176 (1994)). Formalin-fixed, paraffin-embedded human fetal and adult tissues were sectioned, deparaffinized, deproteinized in proteinase K (20 g/ml) for 15 minutes at 37° C, and further processed for *in situ* hybridization as described by Lu and Gillett (1994). A [³³-P] UTP-labeled antisense riboprobe was generated from a PCR product and hybridized at 55° C overnight. The slides were dipped in Kodak NTB2 nuclear track emulsion and exposed for 4 weeks.

In situ hybridization indicated NL5 mRNA expression in adult human breast cancer cells over benign breast epithelium, areas of apocrine metaplasia and sclerosing adenosis. Expression was further observed over infiltrating breast ductal carcinoma cells. In fetal lower limb tissue, high

expression was found at sites of enchondral bone formation, in osteocytes and in periosteum/perichondrium of developing bones. NL5 mRNA was also highly expressed in osteocytes and in periosteum/perichondrium of developing bones of fetal body wall tissue. This distribution suggests a role in bone formation and differentiation (Figures 9-A and 9-B).

5 *In situ* hybridization for NL8 showed highly organized expression pattern in the developing limb, intestine and body wall, suggesting a distinctive functional role at this site, and potential involvement in angiogenesis and patterning (Figures 10-A and 10-B). This expression pattern is distinct from that of NL1 and NL5.

10 **Example 4**

Expression of NL1, NL5, and NL8 in *E. coli*

15 This example illustrates the preparation of an unglycosylated form of the TIE ligands of the present invention in *E. coli*. The DNA sequence encoding a NL1, NL5 or NL8 ligand (SEQ. ID. NOs: 1, 3, and 5, respectively) is initially amplified using selected PCR primers. The primers should contain restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector. A variety of expression vectors may be employed. The vector will preferably encode an antibiotic resistance gene, an origin of replication, e promoter, and a ribozyme binding site. An example of a suitable vector is pBR322 (derived from *E. coli*; see Bolivar et al., Gene 2:95 (1977)) which contains genes for ampicillin and tetracycline resistance. The vector is digested with
20 restriction enzyme and dephosphorylated. The PCR amplified sequences are then ligated into the vector.

25 The ligation mixture is then used to transform a selected *E. coli* strain, using the methods described in Sambrook et al., supra. Transformants are identified by their ability to grow on LB plates and antibiotic resistant colonies are then selected. Plasmid DNA can be isolated and confirmed by restriction analysis.

 Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a later

scale culture. The cells are then grown to a desired optical density. An inducer, such as IPTG may be added.

After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized protein can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

Example 5

Expression of NL1, NL5 and NL8 in mammalian cells

This example illustrates preparation of a glycosylated form of the NL1, NL5 and NL8 ligands by recombinant expression in mammalian cells.

The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector. Optionally, the NL1, NL5 and NL8 DNA is ligated into pRK5 with selected restriction enzymes to allow insertion of the NL1, NL5 and NL8 DNA using ligation methods such as described in Sambrook et al., *supra*. The resulting vector is called pRK5-NL1, NL5 and NL8, respectively.

In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 µg pRK5-NL1, NL5 and NL8 DNA is mixed with about 1 µg DNA encoding the VA RNA gene [Thimmappaya et al., *Cell*, 31:543 (1982)] and dissolved in 500 µl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl₂. To this mixture is added, dropwise, 500 µl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM NaPO₄, and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 µCi/ml ³⁵S-cysteine and 200 µCi/ml

³⁵S-methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of NL1, NL5 and NL8 polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, NL1, NL5 and NL8 may be introduced into 293 cells transiently using the dextran sulfate method described by Sompanyrac et al., Proc. Natl. Acad. Sci., 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 µg pRK5-NL1, NL5 and NL8 DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 µg/ml bovine insulin and 0.1 µg/ml bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed NL1, NL5 and NL8 can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

In another embodiment, NL1, NL5 and NL8 can be expressed in CHO cells. The pRK5-NL1, NL5 and NL8 can be transfected into CHO cells using known reagents such as CaPO₄ or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as ³⁵S-methionine. After determining the presence of NL1, NL5 and NL8 polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed NL1, NL5 and NL8 can then be concentrated and purified by any selected method.

Epitope-tagged NL1, NL5 and NL8 may also be expressed in host CHO cells. NL1, NL5 and NL8 may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into a Baculovirus expression vector. The poly-his tagged NL1, NL5 and NL8 insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells

can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged NL1, NL5 and NL8 can then be concentrated and purified by any selected method, such as by Ni²⁺-chelate affinity chromatography.

5

Example 6

Expression of NL1, NL5 and NL8 in yeast

First, yeast expression vectors are constructed for intracellular production or secretion of NL1, NL5 and NL8 from the ADH2/GAPDH promoter. DNA encoding NL1, NL5 and NL8, a selected signal peptide and the promoter is inserted into suitable restriction enzyme sites in the selected plasmid to direct intracellular expression of NL1, NL5 and NL8. For secretion, DNA encoding NL1, NL5 and NL8 can be cloned into the selected plasmid, together with DNA encoding the ADH2/GAPDH promoter, the yeast alpha-factor secretory signal/leader sequence, and linker sequences (if needed) for expression of NL1, NL5 and NL8.

10

15

Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with Coomassie Blue stain.

20

Recombinant NL1, NL5 and NL8 can subsequently be isolated and purified by removing the yeast cells from the fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The concentrate containing NL1, NL5 and NL8 may further be purified using selected column chromatography resins.

Example 7

Expression of NL1, NL2 and NL8 in Baculovirus

25

The following method describes recombinant expression of NL1, NL5 and NL8 in Baculovirus.

The NL1, NL5 and NL8 is fused upstream of an epitope tag contained with a baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, the NL1, NL5 and NL8 or the desired portion of the NL1, NL5 and NL8 (such as the sequence encoding the extracellular domain of a transmembrane protein) is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites. The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

Recombinant baculovirus is generated by co-transfecting the above plasmid and BaculoGold™ virus DNA (Pharmlngen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses are harvested and used for further amplifications. Viral infection and protein expression is performed as described by O'Reilley et al., Baculovirus expression vectors: A laboratory Manual, Oxford: Oxford University Press (1994).

Expressed poly-his tagged NL1, NL5 and NL8 can then be purified, for example, by Ni²⁺-chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert et al., Nature, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM MgCl₂; 0.1 mM EDTA; 10% Glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45 μm filter. A Ni²⁺-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to baseline A₂₈₀ with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which elutes nonspecifically bound protein. After reaching A₂₈₀ baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by

SDS-PAGE and silver staining or western blot with Ni²⁺-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His₁₀-tagged NL1, NL5 and NL8 are pooled and dialyzed against loading buffer.

Alternatively, purification of the IgG tagged (or Fc tagged) NL1, NL5 and NL8 can be performed using known chromatography techniques, including for instance, Protein A or protein G column chromatography.

Example 8

Preparation of Antibodies that bind NL1, NL2 and NL8

This example illustrates preparation of monoclonal antibodies which can specifically bind NL1, NL2 and NL8.

Techniques for producing the monoclonal antibodies are known in the art and are described, for example, in Goding, supra. Immunogens that may be employed include purified ligands of the present invention, fusion proteins containing such ligands, and cells expressing recombinant ligands on the cell surface. Selection of the immunogen can be made by the skilled artisan without undue experimentation.

Mice, such as Balb/c, are immunized with the immunogen emulsified in complete Freund's adjuvant and injected subcutaneously or intraperitoneally in an amount from 1-100 micrograms. Alternatively, the immunogen is emulsified in MPL-TDM adjuvant (Ribi Immunochemical Research, Hamilton, MT) and injected into the animal's hind food pads. The immunized mice are then boosted 10 to 12 days later with additional immunogen emulsified in the selected adjuvant. Thereafter, for several weeks, the mice might also be boosted with additional immunization injections. Serum samples may be periodically obtained from the mice by retro-orbital bleeding for testing ELISA assays to detect the antibodies.

After a suitable antibody titer has been detected, the animals "positive" for antibodies can be injected with a final intravenous injection of the given ligand. Three to four days later, the mice are sacrificed and the spleen cells are harvested. The spleen cells are then fused (using 35% polyethylene glycol) to a selected murine myeloma cell line such as P3X63AgU.1, available from

ATCC, No. CRL 1597. The fusions generate hybridoma cells which can then be plated in 96 well tissue culture plates containing HAT (hypoxanthine, aminopterin, and thymidine) medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells will be screened in an ELISA for reactivity against the antigen.
5 Determination of "positive" hybridoma cells secreting the desired monoclonal antibodies against the TIE ligands herein is well within the skill in the art.

The positive hybridoma cells can be injected intraperitoneal into syngeneic Balb/c mice to produce ascites containing the anti-TIE-ligand monoclonal antibodies. Alternatively, the hybridoma cells can be grown in tissue culture flasks or roller bottles. Purification of the monoclonal antibodies produced in the ascites can be accomplished using ammonium sulfate precipitation, followed by gel
10 exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can be employed.

Deposit of Material

15 As noted before, the following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

| <u>Material</u> | <u>ATCC Dep. No.</u> | <u>Deposit Date</u> |
|-----------------|----------------------|---------------------|
| NL1-DNA | 22779-1130 | |
| NL5-DNA | 28497-1130 | |
| NL8-DNA | 23339-1130 | |

25 These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of the deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to

the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and Commissioner's rules pursuant thereto (including 37 C.F.R. §1.14 with particular reference to 886 OG 683).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The present specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of the invention. The deposit of material herein does not constitute an admission that the written description is inadequate to enable the practice of any aspect of the invention, including the best more thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Godowski, Paul J.
Gurney, Austin L.

(ii) TITLE OF INVENTION: Tie Ligands

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Dreger, Ginger R.
(B) REGISTRATION NUMBER: 33,055
(C) REFERENCE/DOCKET NUMBER: P1130

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 650/225-3216

(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2290 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCTGAGGGG AGGCCCCGAG CCTTTCTGGG GCCTGGGGGA TCCTCTTGCA 50
 CTGGTGGGTG GAGAGAAGCG CCTGCAGCCA ACCAGGGTCA GGCTGTGCTC 100
 ACAGTTTCCT CTGGCGGCAT GTAAAGGCTC CACAAAGGAG TTGGGAGTTC 150
 AAATGAGGCT GCTGCGGACG GCCTGAGGAT GGACCCCAAG CCCTGGACCT 200
 GCCGAGCGTG GCACTGAGGC AGCGGCTGAC GCTACTGTGA GGGAAAGAAG 250
 GTTGTGAGCA GCCCCGAGG ACCCCTGGCC AGCCCTGGCC CCAGCCTCTG 300
 CCGGAGCCCT CTGTGGAGGC AGAGCCAGTG GAGCCCAGTG AGGCAGGGCT 350
 GCTTGGCAGC CACCGGCCTG CAACTCAGGA ACCCCTCCAG AGGCCATGGA 400
 CAGGCTGCCC CGCTGACGGC CAGGGTGAAG CATGTGAGGA GCCGCCCCGG 450
 AGCCAAGCAG GAGGGAAGAG GCTTTCATAG ATTCTATTCA CAAAGAATAA 500
 CCACCATTTT GCAAGGACCA TGAGGCCACT GTGCGTGACA TGCTGGTGGC 550
 TCGGACTGCT GGCTGCCATG GGAGCTGTTG CAGGCCAGGA GGACGGTTTT 600
 GAGGGCACTG AGGAGGGCTC GCCAAGAGAG TTCATTTACC TAAACAGGTA 650

PATENT DOCKET P1130

CAAGCGGGCG GCGAGTCCC AGGACAAGTG CACCTACACC TTCATTGTGC 700

CCCAGCAGCG GGTACCGGGT GCCATCTGCG TCAACTCCAA GGAGCCTGAG 750

5 GTGCTTCTGG AGAACCGAGT GCATAAGCAG GAGCTAGAGC TGCTCAACAA 800

TGAGCTGCTC AAGCAGAAGC GGCAGATCGA GACGCTGCAG CAGCTGGTGG 850

AGGTGGACGG CGGCATTGTG AGCGAGGTGA AGCTGCTGCG CAAGGAGAGC 900

10 CGCAACATGA ACTCGCGGGT CACGCAGCTC TACATGCAGC TCCTGCACGA 950

GATCATCCGC AAGCGGGACA ACGCGTTGGA GCTCTCCCAG CTGGAGAACA 1000

15 GGATCCTGAA CCAGACAGCC GACATGCTGC AGCTGGCCAG CAAGTACAAG 1050

GACCTGGAGC ACAAGTACCA GCACCTGGCC AACTGGCCC ACAACCAATC 1100

AGAGATCATC GCGCAGCTTG AGGAGCACTG CCAGAGGGTG CCCTCGGCCA 1150

20 GGCCCGTCCC CCAGCCACCC CCCGCTGCCC CGCCCCGGGT CTACCAACCA 1200

CCCACCTACA ACCGCATCAT CAACCAGATC TCTACCAACG AGATCCAGAG 1250

25 TGACCAGAAC CTGAAGGTGC TGCCACCCCC TCTGCCCCACT ATGCCCCACTC 1300

TCACCAGCCT CCCATCTTCC ACCGACAAGC CGTCGGGCCC ATGGAGAGAC 1350

TGCCTGCAGG CCCTGGAGGA TGGCCACGAC ACCAGCTCCA TCTACCTGGT 1400

30 GAAGCCGGAG AACACCAACC GCCTCATGCA GGTGTGGTGC GACCAGAGAC 1450

ACGACCCCGG GGGCTGGACC GTCATCCAGA GACGCCTGGA TGGCTCTGTT 1500

35 AACTTCTTCA GGAAGTGGGA GACGTACAAG CAAGGGTTTG GGAACATTGA 1550

CGGCGAATAC TGGCTGGGCC TGGAGAACAT TTACTGGCTG ACGAACCAAG 1600

GCAACTACAA ACTCCTGGTG ACCATGGAGG ACTGGTCCGG CCGCAAAGTC 1650

TTTGCAGAAT ACGCCAGTTT CCGCCTGGAA CCTGAGAGCG AGTATTATAA 1700

5 GCTGCGGCTG GGGCGCTACC ATGGCAATGC GGGTGACTCC TTTACATGGC 1750

ACAACGGCAA GCAGTTCACC ACCCTGGACA GAGATCATGA TGTCTACACA 1800

GGAAACTGTG CCCACTACCA GAAGGGAGGC TGGTGGTATA ACGCCTGTGC 1850

10 CCACTCCAAC CTCAACGGGG TCTGGTACCG CGGGGGCCAT TACCGGAGCC 1900

GCTACCAGGA CGGAGTCTAC TGGGCTGAGT TCCGAGGAGG CTCTTACTCA 1950

15 CTCAAGAAAG TGGTGATGAT GATCCGACCG AACCCCAACA CCTTCCACTA 2000

AGCCAGCTCC CCCTCCTGAC CTCTCGTGGC CATTGCCAGG AGCCCACCCT 2050

GGTCACGCTG GCCACAGCAC AAAGAACAAC TCCTCACCAG TTCATCCTGA 2100

20 GGCTGGGAGG ACCGGGATGC TGGATTCTGT TTTCCGAAGT CACTGCAGCG 2150

GATGATGGAA CTGAATCGAT ACGGTGTTTT CTGTCCCTCC TACTTTCCTT 2200

25 CACACCAGAC AGCCCCTCAT GTCTCCAGGA CAGGACAGGA CTACAGACAA 2250

CTCTTTCTTT AAATAAATTA AGTCTCTACA ATAAAAAAAA 2290

(2) INFORMATION FOR SEQ ID NO:2:

- 30
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 493 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Arg | Pro | Leu | Cys | Val | Thr | Cys | Trp | Trp | Leu | Gly | Leu | Leu | Ala |
| 1 | | | | 5 | | | | | 10 | | | | | 15 |
| Ala | Met | Gly | Ala | Val | Ala | Gly | Gln | Glu | Asp | Gly | Phe | Glu | Gly | Thr |
| | | | | 20 | | | | | 25 | | | | | 30 |
| Glu | Glu | Gly | Ser | Pro | Arg | Glu | Phe | Ile | Tyr | Leu | Asn | Arg | Tyr | Lys |
| | | | | 35 | | | | | 40 | | | | | 45 |
| Arg | Ala | Gly | Glu | Ser | Gln | Asp | Lys | Cys | Thr | Tyr | Thr | Phe | Ile | Val |
| | | | | 50 | | | | | 55 | | | | | 60 |
| Pro | Gln | Gln | Arg | Val | Thr | Gly | Ala | Ile | Cys | Val | Asn | Ser | Lys | Glu |
| | | | | 65 | | | | | 70 | | | | | 75 |
| Pro | Glu | Val | Leu | Leu | Glu | Asn | Arg | Val | His | Lys | Gln | Glu | Leu | Glu |
| | | | | 80 | | | | | 85 | | | | | 90 |
| Leu | Leu | Asn | Asn | Glu | Leu | Leu | Lys | Gln | Lys | Arg | Gln | Ile | Glu | Thr |
| | | | | 95 | | | | | 100 | | | | | 105 |
| Leu | Gln | Gln | Leu | Val | Glu | Val | Asp | Gly | Gly | Ile | Val | Ser | Glu | Val |
| | | | | 110 | | | | | 115 | | | | | 120 |
| Lys | Leu | Leu | Arg | Lys | Glu | Ser | Arg | Asn | Met | Asn | Ser | Arg | Val | Thr |
| | | | | 125 | | | | | 130 | | | | | 135 |
| Gln | Leu | Tyr | Met | Gln | Leu | Leu | His | Glu | Ile | Ile | Arg | Lys | Arg | Asp |
| | | | | 140 | | | | | 145 | | | | | 150 |
| Asn | Ala | Leu | Glu | Leu | Ser | Gln | Leu | Glu | Asn | Arg | Ile | Leu | Asn | Gln |
| | | | | 155 | | | | | 160 | | | | | 165 |
| Thr | Ala | Asp | Met | Leu | Gln | Leu | Ala | Ser | Lys | Tyr | Lys | Asp | Leu | Glu |
| | | | | 170 | | | | | 175 | | | | | 180 |

PATENT DOCKET P1130

His Lys Tyr Gln His Leu Ala Thr Leu Ala His Asn Gln Ser Glu
185 190 195

Ile Ile Ala Gln Leu Glu Glu His Cys Gln Arg Val Pro Ser Ala
200 205 210

Arg Pro Val Pro Gln Pro Pro Pro Ala Ala Pro Pro Arg Val Tyr
215 220 225

Gln Pro Pro Thr Tyr Asn Arg Ile Ile Asn Gln Ile Ser Thr Asn
230 235 240

Glu Ile Gln Ser Asp Gln Asn Leu Lys Val Leu Pro Pro Pro Leu
245 250 255

Pro Thr Met Pro Thr Leu Thr Ser Leu Pro Ser Ser Thr Asp Lys
260 265 270

Pro Ser Gly Pro Trp Arg Asp Cys Leu Gln Ala Leu Glu Asp Gly
275 280 285

His Asp Thr Ser Ser Ile Tyr Leu Val Lys Pro Glu Asn Thr Asn
290 295 300

Arg Leu Met Gln Val Trp Cys Asp Gln Arg His Asp Pro Gly Gly
305 310 315

Trp Thr Val Ile Gln Arg Arg Leu Asp Gly Ser Val Asn Phe Phe
320 325 330

Arg Asn Trp Glu Thr Tyr Lys Gln Gly Phe Gly Asn Ile Asp Gly
335 340 345

Glu Tyr Trp Leu Gly Leu Glu Asn Ile Tyr Trp Leu Thr Asn Gln
350 355 360

Gly Asn Tyr Lys Leu Leu Val Thr Met Glu Asp Trp Ser Gly Arg
365 370 375

Lys Val Phe Ala Glu Tyr Ala Ser Phe Arg Leu Glu Pro Glu Ser
380 385 390

Glu Tyr Tyr Lys Leu Arg Leu Gly Arg Tyr His Gly Asn Ala Gly
395 400 405

Asp Ser Phe Thr Trp His Asn Gly Lys Gln Phe Thr Thr Leu Asp
410 415 420

Arg Asp His Asp Val Tyr Thr Gly Asn Cys Ala His Tyr Gln Lys
425 430 435

Gly Gly Trp Trp Tyr Asn Ala Cys Ala His Ser Asn Leu Asn Gly
440 445 450

Val Trp Tyr Arg Gly Gly His Tyr Arg Ser Arg Tyr Gln Asp Gly
455 460 465

Val Tyr Trp Ala Glu Phe Arg Gly Gly Ser Tyr Ser Leu Lys Lys
470 475 480

Val Val Met Met Ile Arg Pro Asn Pro Asn Thr Phe His
485 490 493

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3355 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

PATENT DOCKET P1130

GCAGCTGGTT ACTGCATTTTC TCCATGTGGC AGACAGAGCA AAGCCACAAC 50

GCTTTCTCTG CTGGATTAAA GACGGCCCAC AGACCAGAAC TTCCACTATA 100

5 CTA CTACTTAAAA TTACATAGGT GGCTTGTCAG ATTCAATTGA TTAGTATTGT 150

AAAAGGAAAA AGAAGTTCCT TCTTACAGCT TGGATTCAAC GGTCCAAAAAC 200

AAAAATGCAG CTGCCATTAA AGTCTCAGAT GAACAACTT CTACACTGAT 250

10 TTTTAAAAATC AAGAATAAGG GCAGCAAGTT TCTGGATTCA CTGAATCAAC 300

AGACACAAAA AGCTGGCAAT ATAGCAACTA TGAAGAGAAA AGCTACTAAT 350

15 AAAATTAACC CAACGCATAG AAGACTTTTT TTTCTCTTCT AAAACAAC 400

AAGTAAAGAC TTAAATTTAA ACACATCATT TTACAACCTC ATTTCAAAAT 450

GAAGACTTTTT ACCTGGACCC TAGGTGTGCT ATTCTTCCTA CTAGTGGACA 500

20 CTGGACATTG CAGAGGTGGA CAATTCAAAA TTAATAAAAT AAACCAGAGA 550

AGATACCCTC GTGCCACAGA TGGTAAAGAG GAAGCAAAGA AATGTGCATA 600

25 CACATTCTCTG GTACCTGAAC AAAGAATAAC AGGGCCAATC TGTGTCAACA 650

CCAAGGGGCA AGATGCAAGT ACCATTAAAG ACATGATCAC CAGGATGGAC 700

CTTGAAAACC TGAAGGATGT GCTCTCCAGG CAGAAGCGGG AGATAGATGT 750

30 TCTGCAACTG GTGGTGGATG TAGATGGAAC CATTGTGAAT GAGGTAAAGC 800

TGCTGAGAAA GGAAAGCCGT AACATGAACT CTCGTGTTAC TCAACTCTAT 850

35 ATGCAATTAT TACATGAGAT TATCCGTAAG AGGGATAATT CACTTGAAC 900

TTCCCAACTG GAAAACAAAA TCCTCAATGT CACCACAGAA ATGTTGAAGA 950

PATENT DOCKET P1130

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5 GAGGATATTT TCCCGACAAG ACACCCATGT GTCTCCCCCA CTTGTCCAGG 1100

TGGTGCCACA ACATATTCCT AACAGCCAAC AGTATACTCC TGGTCTGCTG 1150

GGAGGTAACG AGATTTCAGAG GGATCCAGGT TATCCCAGAG ATTTAATGCC 1200

10 ACCACCTGAT CTGGCAACTT CTCCCACCAA AAGCCCTTTC AAGATACCAC 1250

CGGTAACTTT CATCAATGAA GGACCATTCA AAGACTGTCA GCAAGCAAAA 1300

15 GAAGCTGGGC ATTCGGTCAG TGGGATTTAT ATGATTAAAC CTGAAAACAG 1350

CAATGGACCA ATGCAGTTAT GGTGTGAAAA CAGTTTGGAC CCTGGGGGTT 1400

GGACTGTTAT TCAGAAAAGA ACAGACGGCT CTGTCAACTT CTTTCAGAAAT 1450

20 TGGGAAAATT ATAAGAAAGG GTTTGGAAAC ATTGACGGAG AATACTGGCT 1500

TGGACTGGAA AATATCTATA TGCTTAGCAA TCAAGATAAT TACAAGTTAT 1550

25 TGATTGAATT AGAAGACTGG AGTGATAAAA AAGTCTATGC AGAATACAGC 1600

AGCTTTCGTC TGGAACCTGA AAGTGAATTC TATAGACTGC GCCTGGGAAC 1650

TTACCAGGGA AATGCAGGGG ATTCTATGAT GTGGCATAAT GGTAACAAT 1700

30 TCACCACACT GGACAGAGAT AAAGATATGT ATGCAGGAAA CTGCGCCAC 1750

TTTCATAAAG GAGGCTGGTG GTACAATGCC TGTGCACATT CTAACCTAAA 1800

35 TGGAGTATGG TACAGAGGAG GCCATTACAG AAGCAAGCAC CAAGATGGAA 1850

TTTTCTGGGC CGAATACAGA GGCGGGTCAT ACTCCTTAAG AGCAGTTCAG 1900

PATENT DOCKET P1130

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CACAGAACTT TGTACTTTTC AGCTCTTAAA AATGTAAATG TTACATGTAT 2000

5 ATTACTTGGC ACAATTTATT TCTACACAGA AAGTTTTTAA AATGAATTTT 2050

ACCGTAACTA TAAAAGGGAA CCTATAAATG TAGTTTCATC TGTCGTCAAT 2100

10 TACTGCAGAA AATTATGTGT ATCCACAACC TAGTTATTTT AAAAATTATG 2150

TTGACTAAAT ACAAAGTTTG TTTTCTAAAA TGTAAATATT TGCCACAATG 2200

TAAAGCAAAT CTTAGCTATA TTTTAAATCA TAAATAACAT GTTCAAGATA 2250

15 CTTAACAATT TATTTAAAT CTAAGATTGC TCTAACGTCT AGTGAAAAAA 2300

ATATTTTTTA AATTTAGCC AAATAATGCA TTTTATTTTA TAAAAATACA 2350

GACAGAAAAT TAGGGAGAAA CTTCTAGTTT TGCCAATAGA AAATGTTCTT 2400

20 CCATTGAATA AAAGTTATTT CAAATTGAAT TTGTGCCTTT CACACGTAAT 2450

GATTAAATCT GAATTCCTAA TAATATATCC TATGCTGATT TTCCCAAAC 2500

25 ATGACCCATA GTATTAAATA CATATCATTT TTAATAATAA AAAAAACCC 2550

AAAAATAATG CATGCATAAT TTAAATGGTC AATTTATAAA GACAAATCTA 2600

TGAATGAATT TTTCAGTGTT ATCTTCATAT GATATGCTGA ACACCAAAT 2650

30 CTCCAGAAAT GCATTTTATG TAGTTCTAAA ATCAGCAAAA TATTGGTATT 2700

ACAAAAATGC AGAATATTTA GTGTGCTACA GATCTGAATT ATAGTTCTAA 2750

35 TTTATTATT ATTTTTTTCT AATTTACTGA TCTTACTACT ACAAAGAAAA 2800

AAAAACCCAA CCCATCTGCA ATTCAAATCA GAAAGTTTGG ACAGCTTTAC 2850

AAGTATTAGT GCATGCTCAG AACAGGTGGG ACTAAAACAA ACTCAAGGAA 2900

CTGTTGGCTG TTTTCCCGAT ACTGAGAATT CAACAGCTCC AGAGCAGAAG 2950

5 CCACAGGGGC ATAGCTTAGT CCAAAGTGT AATTTCATTT TACAGTGTAT 3000

GTAACGCTTA GTCTCACAGT GTCTTTAACT CATCTTTGCA ATCAACAAC 3050

TTACTAGTGA CTTTCTGGAA CAATTTCCCTT TCAGGAATAC ATATTCACTG 3100

10 CTTAGAGGTG ACCTTGCCTT AATATATTTG TGAAGTTAAA ATTTTAAAGA 3150

TAGCTCATGA AACTTTTGCT TAAGCAAAAA GAAAACCTCG AATTGAAATG 3200

15 TGTGAGGCAA ACTATGCATG GGAATAGCTT AATGTGAAGA TAATCATTTG 3250

GACAACTCAA ATCCATCAAC ATGACCAATG TTTTTCATCT GCCACATCTC 3300

AAAAATAAAC TTCTGGTGAA ACAAATTAAA CAAAATATCC AAACCTCAAA 3350

20 AAAAA 3355

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 491 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Lys | Thr | Phe | Thr | Trp | Thr | Leu | Gly | Val | Leu | Phe | Phe | Leu | Leu |
| 1 | | | | 5 | | | | 10 | | | | | 15 | |
| Val | Asp | Thr | Gly | His | Cys | Arg | Gly | Gly | Gln | Phe | Lys | Ile | Lys | Lys |
| | | | | 20 | | | | 25 | | | | | 30 | |

PATENT DOCKET P1130

Ile Asn Gln Arg Arg Tyr Pro Arg Ala Thr Asp Gly Lys Glu Glu
35 40 45

Ala Lys Lys Cys Ala Tyr Thr Phe Leu Val Pro Glu Gln Arg Ile
5 50 55 60

Thr Gly Pro Ile Cys Val Asn Thr Lys Gly Gln Asp Ala Ser Thr
65 70 75

Ile Lys Asp Met Ile Thr Arg Met Asp Leu Glu Asn Leu Lys Asp
10 80 85 90

Val Leu Ser Arg Gln Lys Arg Glu Ile Asp Val Leu Gln Leu Val
95 100 105

Val Asp Val Asp Gly Asn Ile Val Asn Glu Val Lys Leu Leu Arg
110 115 120

Lys Glu Ser Arg Asn Met Asn Ser Arg Val Thr Gln Leu Tyr Met
125 130 135

Gln Leu Leu His Glu Ile Ile Arg Lys Arg Asp Asn Ser Leu Glu
140 145 150

Leu Ser Gln Leu Glu Asn Lys Ile Leu Asn Val Thr Thr Glu Met
155 160 165

Leu Lys Met Ala Thr Arg Tyr Arg Glu Leu Glu Val Lys Tyr Ala
170 175 180

Ser Leu Thr Asp Leu Val Asn Asn Gln Ser Val Met Ile Thr Leu
185 190 195

Leu Glu Glu Gln Cys Leu Arg Ile Phe Ser Arg Gln Asp Thr His
200 205 210

PATENT DOCKET P1130

Val Ser Pro Pro Leu Val Gln Val Val Pro Gln His Ile Pro Asn
215 220 225

Ser Gln Gln Tyr Thr Pro Gly Leu Leu Gly Gly Asn Glu Ile Gln
230 235 240

Arg Asp Pro Gly Tyr Pro Arg Asp Leu Met Pro Pro Pro Asp Leu
245 250 255

Ala Thr Ser Pro Thr Lys Ser Pro Phe Lys Ile Pro Pro Val Thr
260 265 270

Phe Ile Asn Glu Gly Pro Phe Lys Asp Cys Gln Gln Ala Lys Glu
275 280 285

Ala Gly His Ser Val Ser Gly Ile Tyr Met Ile Lys Pro Glu Asn
290 295 300

Ser Asn Gly Pro Met Gln Leu Trp Cys Glu Asn Ser Leu Asp Pro
305 310 315

Gly Gly Trp Thr Val Ile Gln Lys Arg Thr Asp Gly Ser Val Asn
320 325 330

Phe Phe Arg Asn Trp Glu Asn Tyr Lys Lys Gly Phe Gly Asn Ile
335 340 345

Asp Gly Glu Tyr Trp Leu Gly Leu Glu Asn Ile Tyr Met Leu Ser
350 355 360

Asn Gln Asp Asn Tyr Lys Leu Leu Ile Glu Leu Glu Asp Trp Ser
365 370 375

Asp Lys Lys Val Tyr Ala Glu Tyr Ser Ser Phe Arg Leu Glu Pro
380 385 390

Glu Ser Glu Phe Tyr Arg Leu Arg Leu Gly Thr Tyr Gln Gly Asn
395 400 405

Ala Gly Asp Ser Met Met Trp His Asn Gly Lys Gln Phe Thr Thr
410 415 420

Leu Asp Arg Asp Lys Asp Met Tyr Ala Gly Asn Cys Ala His Phe
425 430 435

His Lys Gly Gly Trp Trp Tyr Asn Ala Cys Ala His Ser Asn Leu
440 445 450

Asn Gly Val Trp Tyr Arg Gly Gly His Tyr Arg Ser Lys His Gln
455 460 465

Asp Gly Ile Phe Trp Ala Glu Tyr Arg Gly Gly Ser Tyr Ser Leu
470 475 480

Arg Ala Val Gln Met Met Ile Lys Pro Ile Asp
485 490 491

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1780 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCTCAGAGG CCCCACTGGA CCCTCGGCTC TTCCTTGGAC TTCTTGTTGTG 50

TTCTGTGAGC TTCGCTGGAT TCAGGGTCTT GGGCATCAGA GGTGAGAGGG 100

TGGGAAGGTC CGCCGCGATG GGGAAGCCCT GGCTGCGTGC GCTACAGCTG 150

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CTGCTCCTGC TGGGCGCGTC GTGGGCGCGG GCGGGCGCCC CGCGCTGCAC 200

CTACACCTTC GTGCTGCCCC CGCAGAAAGTT CACGGGCGCT GTGTGCTGGA 250

5 GCGGCCCCGC ATCCACGCGG GCGACGCCCC AGGCCGCCAA CGCCAGCGAG 300

CTGGCGGCGC TGCGCATGCG CGTCGGCCGC CACGAGGAGC TGTTACGCGA 350

GCTGCAGAGG CTGGCGGCGG CCGACGGCGC CGTGGCCGGC GAGGTGCGCG 400

10 CGCTGCGCAA GGAGAGCCGC GGCCTGAGCG CGCGCCTGGG CCAGTTGCGC 450

GCGCAGCTGC AGCACGAGGC GGGGCCCCGG GCGGGCCCCG GGGCGGATCT 500

15 GGGGGCGGAG CCTGCCGCGG CGCTGGCGCT GCTCGGGGAG CGCGTGCTCA 550

ACGCGTCCGC CGAGGCTCAG CGCGCAGCCG CCCGGTTCCA CCAGCTGGAC 600

GTCAAGTTCC GCGAGCTGGC GCAGCTCGTC ACCCAGCAGA GCAGTCTCAT 650

20 CGCCCGCCTG GAGCGCCTGT GCCCGGGAGG CGCGGGCGGG CAGCAGCAGG 700

TCCTGCCGCC ACCCCCACTG GTGCCTGTGG TTCCGGTCCG TCTTGTGGGT 750

25 AGCACCAGTG ACACCAGTAG GATGCTGGAC CCAGCCCCAG AGCCCCAGAG 800

AGACCAGACC CAGAGACAGC AGGAGCCCAT GGCTTCTCCC ATGCCTGCAG 850

GTCACCCTGC GGTCCCCACC AAGCCTGTGG GCCCGTGGCA GGATTGTGCA 900

30 GAGGCCCGCC AGGCAGGCCA TGAACAGAGT GGAGTGTATG AACTGCGAGT 950

GGGCCGTCAC GTAGTGTGAG TATGGTGTGA GCAGCAACTG GAGGGTGGAG 1000

35 GCTGGACTGT GATCCAGCGG AGGCAAGATG GTTCAGTCAA CTTCTTCACT 1050

ACCTGGCAGC ACTATAAGGC GGGCTTTGGG CGGCCAGACG GAGAATACTG 1100

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GCTGGGCCTT GAACCCGTGT ATCAGCTGAC CAGCCGTGGG GACCATGAGC 1150

TGCTGGTTCT CCTGGAGGAC TGGGGGGGCC GTGGAGCACG TGCCCACTAT 1200

5 GATGGCTTCT CCCTGGAACC CGAGAGCGAC CACTACCGCC TCGGGCTTGG 1250

CCAGTACCAT GGTGATGCTG GAGACTCTCT TTCCTGGCAC AATGACAAGC 1300

10 CCTTCAGCAC CGTGGATAGG GACCGAGACT CCTATTCTGG TAACTGTGCC 1350

CTGTACCAGC GGGGAGGCTG GTGGTACCAT GCCTGTGCCC ACTCCAACCT 1400

CAACGGTGTG TGGCACCACG GCGGCCACTA CCGAAGCCGC TACCAGGATG 1450

15 GTGTCTACTG GGCTGAGTTT CGTGGTGGGG CATATTCTCT CAGGAAGGCC 1500

GCCATGCTCA TTCGGCCCCCT GAAGCTGTGA CTCTGTGTTC CTCTGTCCCC 1550

20 TAGGCCCTAG AGGACATTGG TCAGCAGGAG CCCAAGTTGT TCTGGCCACA 1600

CCTTCTTTGT GGCTCAGTGC CAATGTGTCC CACAGAACTT CCCACTGTGG 1650

ATCTGTGACC CTGGGCGCTG AAAATGGGAC CCAGGAATCC CCCCCGTCAA 1700

25 TATCTTGGCC TCAGATGGCT CCCCAAGGTC ATTCATATCT CGGTTTGAGC 1750

TCATATCTTA TAATAACACA AAGTAGCCAC 1780

(2) INFORMATION FOR SEQ ID NO:6:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 470 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Gly | Lys | Pro | Trp | Leu | Arg | Ala | Leu | Gln | Leu | Leu | Leu | Leu | Leu |
| 1 | | | | 5 | | | | | 10 | | | | | 15 |
| Gly | Ala | Ser | Trp | Ala | Arg | Ala | Gly | Ala | Pro | Arg | Cys | Thr | Tyr | Thr |
| | | | | 20 | | | | | 25 | | | | | 30 |
| Phe | Val | Leu | Pro | Pro | Gln | Lys | Phe | Thr | Gly | Ala | Val | Cys | Trp | Ser |
| | | | | 35 | | | | | 40 | | | | | 45 |
| Gly | Pro | Ala | Ser | Thr | Arg | Ala | Thr | Pro | Glu | Ala | Ala | Asn | Ala | Ser |
| | | | | 50 | | | | | 55 | | | | | 60 |
| Glu | Leu | Ala | Ala | Leu | Arg | Met | Arg | Val | Gly | Arg | His | Glu | Glu | Leu |
| | | | | 65 | | | | | 70 | | | | | 75 |
| Leu | Arg | Glu | Leu | Gln | Arg | Leu | Ala | Ala | Ala | Asp | Gly | Ala | Val | Ala |
| | | | | 80 | | | | | 85 | | | | | 90 |
| Gly | Glu | Val | Arg | Ala | Leu | Arg | Lys | Glu | Ser | Arg | Gly | Leu | Ser | Ala |
| | | | | 95 | | | | | 100 | | | | | 105 |
| Arg | Leu | Gly | Gln | Leu | Arg | Ala | Gln | Leu | Gln | His | Glu | Ala | Gly | Pro |
| | | | | 110 | | | | | 115 | | | | | 120 |
| Gly | Ala | Gly | Pro | Gly | Ala | Asp | Leu | Gly | Ala | Glu | Pro | Ala | Ala | Ala |
| | | | | 125 | | | | | 130 | | | | | 135 |
| Leu | Ala | Leu | Leu | Gly | Glu | Arg | Val | Leu | Asn | Ala | Ser | Ala | Glu | Ala |
| | | | | 140 | | | | | 145 | | | | | 150 |
| Gln | Arg | Ala | Ala | Ala | Arg | Phe | His | Gln | Leu | Asp | Val | Lys | Phe | Arg |
| | | | | 155 | | | | | 160 | | | | | 165 |
| Glu | Leu | Ala | Gln | Leu | Val | Thr | Gln | Gln | Ser | Ser | Leu | Ile | Ala | Arg |
| | | | | 170 | | | | | 175 | | | | | 180 |

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Leu Glu Arg Leu Cys Pro Gly Gly Ala Gly Gly Gln Gln Gln Val
 185 190 195

Leu Pro Pro Pro Pro Leu Val Pro Val Val Pro Val Arg Leu Val
 200 205 210

Gly Ser Thr Ser Asp Thr Ser Arg Met Leu Asp Pro Ala Pro Glu
 215 220 225

Pro Gln Arg Asp Gln Thr Gln Arg Gln Gln Glu Pro Met Ala Ser
 230 235 240

Pro Met Pro Ala Gly His Pro Ala Val Pro Thr Lys Pro Val Gly
 245 250 255

Pro Trp Gln Asp Cys Ala Glu Ala Arg Gln Ala Gly His Glu Gln
 260 265 270

Ser Gly Val Tyr Glu Leu Arg Val Gly Arg His Val Val Ser Val
 275 280 285

Trp Cys Glu Gln Gln Leu Glu Gly Gly Gly Trp Thr Val Ile Gln
 290 295 300

Arg Arg Gln Asp Gly Ser Val Asn Phe Phe Thr Thr Trp Gln His
 305 310 315

Tyr Lys Ala Gly Phe Gly Arg Pro Asp Gly Glu Tyr Trp Leu Gly
 320 325 330

Leu Glu Pro Val Tyr Gln Leu Thr Ser Arg Gly Asp His Glu Leu
 335 340 345

Leu Val Leu Leu Glu Asp Trp Gly Gly Arg Gly Ala Arg Ala His
 350 355 360

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Tyr Asp Gly Phe Ser Leu Glu Pro Glu Ser Asp His Tyr Arg Leu
365 370 375

Arg Leu Gly Gln Tyr His Gly Asp Ala Gly Asp Ser Leu Ser Trp
380 385 390

His Asn Asp Lys Pro Phe Ser Thr Val Asp Arg Asp Arg Asp Ser
395 400 405

Tyr Ser Gly Asn Cys Ala Leu Tyr Gln Arg Gly Gly Trp Trp Tyr
410 415 420

His Ala Cys Ala His Ser Asn Leu Asn Gly Val Trp His His Gly
425 430 435

Gly His Tyr Arg Ser Arg Tyr Gln Asp Gly Val Tyr Trp Ala Glu
440 445 450

Phe Arg Gly Gly Ala Tyr Ser Leu Arg Lys Ala Ala Met Leu Ile
455 460 465

Arg Pro Leu Lys Leu
470

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGACGAAC CAAGGCAACT ACAAACCTCCT GGT 33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCGGCCGGA CCAGTCCTCC ATGGTCACCA GGAGTTTGTA G 41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGGTGAAC TGCTTGCCGT TGTGCCATGT AAA 33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGTTATCC CAGAGATTTA ATGCCACCA 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTGGTGGGAG AAGTTGCCAG ATCAGGTGGT GGCA 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTCACACCAT AACTGCATTG GTCCA 25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACGTAGTTCC AGTATGGTGT GAGCAGCAAC TGGA 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGTCCAGCCT CCACCCTCCA GTTGCT 26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCCCAGTCCT CCAGGAGAAC CAGCA 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2042 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

5 GCGGACGCGT GGGTGAAATT GAAAATCAAG ATAAAAATGT TCACAATTAA 50
GCTCCTTCTT TTTATTGTTC CTCTAGTTAT TTCCTCCAGA ATTGATCAAG 100
ACAATTCATC ATTTGATTCT CTATCTCCAG AGCCAAAATC AAGATTTGCT 150
10 ATGTTAGACG ATGTAAAAAT TTTAGCCAAT GGCCTCCTTC AGTTGGGACA 200
TGGTCTTAAA GACTTTGTCC ATAAGACGAA GGGCCAAATT AATGACATAT 250
TTCAAAAAC TCAACATATTT GATCAGTCTT TTTATGATCT ATCGCTGCAA 300
15 ACCAGTGAAG TCAAAGAAGA AGAAAAGGAA CTGAGAAGAA CTACATATAA 350
ACTACAAGTC AAAAATGAAG AGGTAAAGAA TATGTCACTT GAACTCAACT 400
20 CAAAACCTGA AAGCCTCCTA GAAGAAAAAA TTCTACTTCA ACAAAAAGTG 450
AAATATTTAG AAGAGCAACT AACTAACTTA ATTCAAAATC AACCTGAAAC 500
TCCAGAACAC CCAGAAGTAA CTTCACTTAA AACTTTTGTA GAAAAACAAG 550
25 ATAATAGCAT CAAAGACCTT CTCCAGACCG TGGAAGACCA ATATAAACAA 600
TTAAACCAAC AGCATAGTCA AATAAAAGAA ATAGAAAATC AGCTCAGAAG 650
30 GACTAGTATT CAAGAACCCA CAGAAATTTT TCTATCTTCC AAGCCAAGAG 700
CACCAAGAAC TACTCCCTTT CTTCAGTTGA ATGAAATAAG AAATGTAAAA 750
CATGATGGCA TTCCTGCTGA ATGTACCACC ATTTATAACA GAGGTGAACA 800
35 TACAAGTGGC ATGTATGCCA TCAGACCCAG CAACTCTCAA GTTTTTCATG 850

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TCTACTGTGA TGTTATATCA GGTAGTCCAT GGACATTAAT TCAACATCGA 900

ATAGATGGAT CACAAAACCTT CAATGAAACG TGGGAGAACT ACAAATATGG 950

5 TTTTGGGAGG CTTGATGGAG AATTTTGGTT GGGCCTAGAG AAGATATACT 1000

CCATAGTGAA GCAATCTAAT TATGTTTTAC GAATTGAGTT GGAAGACTGG 1050

AAAGACAACA AACATTATAT TGAATATTCT TTTTACTTGG GAAATCACGA 1100

10 AACCAACTAT ACGCTACATC TAGTTGCGAT TACTGGCAAT GTCCCCAATG 1150

CAATCCCGGA AAACAAAGAT TTGGTGTTTT CTACTTGGGA TCACAAAGCA 1200

15 AAAGGACACT TCAACTGTCC AGAGGGTTAT TCAGGAGGCT GGTGGTGGCA 1250

TGATGAGTGT GGAGAAAACA ACCTAAATGG TAAATATAAC AAACCAAGAG 1300

CAAATCTAA GCCAGAGAGG AGAAGAGGAT TATCTTGGAA GTCTCAAAAT 1350

20 GGAAGGTTAT ACTCTATAAA ATCAACCAAA ATGTTGATCC ATCCAACAGA 1400

TTCAGAAAGC TTTGAATGAA CTGAGGCAAT TTAAAGGCAT ATTTAACCAT 1450

25 TAACTCATTC CAAGTTAATG TGGTCTAATA ATCTGGTATA AATCCTTAAG 1500

AGAAAGCTTG AGAAATAGAT TTTTTTTATC TTAAAGTCAC TGTCTATTTA 1550

AGATTAAACA TACAATCACA TAACCTTAAA GAATACCGTT TACATTTCTC 1600

30 AATCAAAATT CTTATAATAC TATTTGTTTT AAATTTTGTG ATGTGGGAAT 1650

CAATTTTAGA TGGTCACAAT CTAGATTATA ATCAATAGGT GAACTTATTA 1700

35 AATAACTTTT CTAAATAAAA AATTTAGAGA CTTTATTTT AAAAGGCATC 1750

ATATGAGCTA ATATCACAAC TTTCCCAGTT TAAAAACTA GTACTCTTGT 1800

TAAAACTCTA AACTTGACTA AATACAGAGG ACTGGTAATT GTACAGTTCT 1850

TAAATGTTGT AGTATTAATT TCAAAACTAA AAATCGTCAG CACAGAGTAT 1900

5 GTGTAAAAAT CTGTAATACA AATTTTAA CTGATGCTTC ATTTTGCTAC 1950

AAAATAATTT GGAGTAAATG TTTGATATGA TTTATTTATG AAACCTAATG 2000

AAGCAGAATT AAATACTGTA TTAAAATAAG TTCGCTGTCT TT 2042

10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 460 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Phe Thr Ile Lys Leu Leu Leu Phe Ile Val Pro Leu Val Ile
1 5 10 15

Ser Ser Arg Ile Asp Gln Asp Asn Ser Ser Phe Asp Ser Leu Ser
20 25 30

Pro Glu Pro Lys Ser Arg Phe Ala Met Leu Asp Asp Val Lys Ile
35 40 45

Leu Ala Asn Gly Leu Leu Gln Leu Gly His Gly Leu Lys Asp Phe
50 55 60

Val His Lys Thr Lys Gly Gln Ile Asn Asp Ile Phe Gln Lys Leu
65 70 75

Asn Ile Phe Asp Gln Ser Phe Tyr Asp Leu Ser Leu Gln Thr Ser
80 85 90

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Glu Ile Lys Glu Glu Glu Lys Glu Leu Arg Arg Thr Thr Tyr Lys
95 100 105

Leu Gln Val Lys Asn Glu Glu Val Lys Asn Met Ser Leu Glu Leu
110 115 120

Asn Ser Lys Leu Glu Ser Leu Leu Glu Glu Lys Ile Leu Leu Gln
125 130 135

Gln Lys Val Lys Tyr Leu Glu Glu Gln Leu Thr Asn Leu Ile Gln
140 145 150

Asn Gln Pro Glu Thr Pro Glu His Pro Glu Val Thr Ser Leu Lys
155 160 165

Thr Phe Val Glu Lys Gln Asp Asn Ser Ile Lys Asp Leu Leu Gln
170 175 180

Thr Val Glu Asp Gln Tyr Lys Gln Leu Asn Gln Gln His Ser Gln
185 190 195

Ile Lys Glu Ile Glu Asn Gln Leu Arg Arg Thr Ser Ile Gln Glu
200 205 210

Pro Thr Glu Ile Ser Leu Ser Ser Lys Pro Arg Ala Pro Arg Thr
215 220 225

Thr Pro Phe Leu Gln Leu Asn Glu Ile Arg Asn Val Lys His Asp
230 235 240

Gly Ile Pro Ala Glu Cys Thr Thr Ile Tyr Asn Arg Gly Glu His
245 250 255

Thr Ser Gly Met Tyr Ala Ile Arg Pro Ser Asn Ser Gln Val Phe
260 265 270

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His Val Tyr Cys Asp Val Ile Ser Gly Ser Pro Trp Thr Leu Ile
275 280 285

Gln His Arg Ile Asp Gly Ser Gln Asn Phe Asn Glu Thr Trp Glu
290 295 300

Asn Tyr Lys Tyr Gly Phe Gly Arg Leu Asp Gly Glu Phe Trp Leu
305 310 315

Gly Leu Glu Lys Ile Tyr Ser Ile Val Lys Gln Ser Asn Tyr Val
320 325 330

Leu Arg Ile Glu Leu Glu Asp Trp Lys Asp Asn Lys His Tyr Ile
335 340 345

Glu Tyr Ser Phe Tyr Leu Gly Asn His Glu Thr Asn Tyr Thr Leu
350 355 360

His Leu Val Ala Ile Thr Gly Asn Val Pro Asn Ala Ile Pro Glu
365 370 375

Asn Lys Asp Leu Val Phe Ser Thr Trp Asp His Lys Ala Lys Gly
380 385 390

His Phe Asn Cys Pro Glu Gly Tyr Ser Gly Gly Trp Trp Trp His
395 400 405

Asp Glu Cys Gly Glu Asn Asn Leu Asn Gly Lys Tyr Asn Lys Pro
410 415 420

Arg Ala Lys Ser Lys Pro Glu Arg Arg Arg Gly Leu Ser Trp Lys
425 430 435

Ser Gln Asn Gly Arg Leu Tyr Ser Ile Lys Ser Thr Lys Met Leu
440 445 450

Ile His Pro Thr Asp Ser Glu Ser Phe Glu
455 460

Claims:

1. An isolated nucleic acid molecule encoding a mammalian TIE ligand,
 - (a) selected from the group consisting of human NL-1 (SEQ. ID. NO: 2), human NL-5 (SEQ. ID. NO: 4), human NL8 (SEQ. ID. NO: 6), and homologs thereof in a non-human mammalian species; or
 - (b) a biologically active functional derivative thereof,provided that if the functional derivative is an amino acid sequence variant, it has at least about 90% sequence identity with the fibrinogen-like domain of a human NL-1, human NL-5 or human NL8 ligand.
2. The isolated nucleic acid molecule of claim 1 which comprises the coding region of SEQ. ID. NO: 1; SEQ. ID. NO: 3; or SEQ. ID. NO: 5.
3. The isolated nucleic acid molecule of claim 1 which comprises the fibrinogen-like domain of SEQ. ID. NO:1; SEQ. ID. NO:3; or SEQ. ID. NO: 5.
4. A vector which comprises a nucleic acid molecule of claim 1.
5. A recombinant host cell transformed with a nucleic acid molecule according to claim 1.
6. The recombinant host cell of claim 5 which is a prokaryotic cell.
7. The recombinant host cell of claim 5 which is a eukaryotic cell.
8. An isolated mammalian TIE ligand,

(a) selected from the group consisting of human NL-1 (SEQ. ID. NO: 2), human NL-5 (SEQ. ID NO: 4), human NL8 (SEQ. ID. NO: 6), and homologs thereof in a non-human mammalian species; or

(b) a biologically active functional derivative thereof,

provided that if the functional derivative is an amino acid sequence variant, it has at least about 90% sequence identity with the fibrinogen-like region of a human NL-1, human NL-5 or human NL-8 ligand.

9. An antibody which specifically binds the TIE ligand according to claim 8.

10. The antibody of claim 9 which is a monoclonal antibody.

11. The antibody of claim 10 which is an antagonist of the TIE-2 receptor.

12. The antibody of claim 10 which is an agonist of the TIE-2 receptor.

13. A composition comprising a TIE ligand according to claim 8 or an antibody according to claim 9, in association with a carrier.

14. A conjugate comprising a TIE ligand according to claim 8 or an antibody according to claim 9, fused to a further therapeutic or cytotoxic agent.

15. The conjugate of claim 14 wherein the further therapeutic agent is a toxin, another TIE ligand, or a member of the vascular endothelial growth factor (VEGF) family.

16. A method for identifying a cell expressing a TIE receptor comprising contacting the cell with a detectably labeled TIE ligand according to claim 8 under conditions permitting the binding of said TIE ligand to the TIE receptor, and monitoring the binding.

17. A method for identifying an antagonist of a TIE receptor, comprising contacting cells expressing the TIE receptor with a TIE ligand according to claim 8 and a test compound, under conditions permitting the binding of said TIE ligand to the TIE receptor, and determining whether the test compound is capable of interfering with the binding of the TIE ligand to the TIE receptor.

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18. A method for imaging the presence of angiogenesis, which comprises administering to a patient a detectably labeled TIE ligand according to claim 8, or antibody agonist according to claim 9 of a TIE receptor, and monitoring angiogenesis.

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19. A method for inhibiting vasculogenesis, comprising administering to a patient an effective amount of a TIE ligand according to claim 8.

20. The method of claim 19 wherein said TIE ligand is a native human NL8 molecule.

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21. A method of inhibiting tumor growth, comprising administering to a patient an effective amount of a TIE ligand according to claim 8.

22. A method for promoting bone development, maturation or growth, comprising administering to a patient in need an effective amount of TIE ligand according to claim 8.

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ABSTRACT

The present invention concerns isolated nucleic acid molecules encoding the novel TIE ligands NL1, NL5 and NL8, the proteins encoded by such nucleic acid molecules, as well as methods
5 and means for making and using such nucleic acid and protein molecules.

08033821.001002

Docket No. P1130

**COMBINED DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

TIE LIGANDS

the specification of which (check one) ☒ is attached hereto or ☐ was filed on as Application Serial No. and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

Priority Claimed
Yes No

| Number | Country | Day/Month/Year Filed |
|--------|---------|----------------------|
|--------|---------|----------------------|

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

| Application Ser. No. | Filing Date |
|----------------------|-------------|
|----------------------|-------------|

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| Application Ser. No. | Filing Date | Status: Patented, Pending, Abandoned |
|----------------------|-------------|--------------------------------------|
|----------------------|-------------|--------------------------------------|

| Application Ser. No. | Filing Date | Status: Patented, Pending, Abandoned |
|----------------------|-------------|--------------------------------------|
|----------------------|-------------|--------------------------------------|

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Walter E. Buting - Reg. No. 23,092
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 Telephone: (650) 225-3216

I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Full name of sole or first inventor

Paul J. Godowski

Inventor's signature

Date

9/19/97

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Inventor's signature

Date

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GCGGACGCGT GGGTGAAATT GAAAATCAAG ATAAAAATGT TCACAATTAA 50
 GCTCCTTCTT TTTATTGTTC CTCTAGTTAT TTCCTCCAGA ATTGATCAAG 100
 ACAATTCATC ATTTGATTCT CTATCTCCAG AGCCAAAATC AAGATTTGCT 150
 ATGTTAGACG ATGTAAAAAT TTTAGCCAAT GGCCTCCTTC AGTTGGGACA 200
 TGGTCTTAAA GACTTTGTCC ATAAGACGAA GGGCCAAATT AATGACATAT 250
 TTCAAAACT CAACATATTT GATCAGTCTT TTTATGATCT ATCGCTGCAA 300
 ACCAGTGAAA TCAAAGAAGA AGAAAAGGAA CTGAGAAGAA CTACATATAA 350
 ACTACAAGTC AAAAATGAAG AGGTAAAGAA TATGTCACTT GAACTCAACT 400
 CAAAACCTGA AAGCCTCCTA GAAGAAAAAA TTCTACTTCA AAAAAAGTG 450
 AAATATTTAG AAGAGCAACT AACTAACTTA ATTCAAATC AACCTGAAAC 500
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 TTAAACCAAC AGCATAGTCA AATAAAAGAA ATAGAAAATC AGCTCAGAAG 650
 GACTAGTATT CAAGAACCCA CAGAAATTTT TCTATCTTCC AAGCCAAGAG 700
 CACCAAGAAC TACTCCCTTT CTTCACTTGA ATGAAATAAG AAATGTAAAA 750
 CATGATGGCA TTCCTGCTGA ATGTACCACC ATTTATAACA GAGGTGAACA 800
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 TTTTGGGAGG CTTGATGGAG AATTTTGGTT GGGCCTAGAG AAGATATACT 1000
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 TGATGAGTGT GGAGAAAACA ACCTAAATGG TAAATATAAC AAACCAAGAG 1300
 CAAAATCTAA GCCAGAGAGG AGAAGAGGAT TATCTTGGA GTCTCAAAAT 1350
 GGAAGGTTAT ACTCTATAAA ATCAACCAAA ATGTTGATCC ATCCAACAGA 1400
 TTCAGAAAGC TTTGAATGAA CTGAGGCAAT TTAAAGGCAT ATTTAACCAT 1450

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Phe | Thr | Ile | Lys | Leu | Leu | Leu | Phe | Ile | Val | Pro | Leu | Val | Ile |
| 1 | | | | 5 | | | | | 10 | | | | | 15 |
| Ser | Ser | Arg | Ile | Asp | Gln | Asp | Asn | Ser | Ser | Phe | Asp | Ser | Leu | Ser |
| | | | | 20 | | | | | 25 | | | | | 30 |
| Pro | Glu | Pro | Lys | Ser | Arg | Phe | Ala | Met | Leu | Asp | Asp | Val | Lys | Ile |
| | | | | 35 | | | | | 40 | | | | | 45 |
| Leu | Ala | Asn | Gly | Leu | Leu | Gln | Leu | Gly | His | Gly | Leu | Lys | Asp | Phe |
| | | | | 50 | | | | | 55 | | | | | 60 |
| Val | His | Lys | Thr | Lys | Gly | Gln | Ile | Asn | Asp | Ile | Phe | Gln | Lys | Leu |
| | | | | 65 | | | | | 70 | | | | | 75 |
| Asn | Ile | Phe | Asp | Gln | Ser | Phe | Tyr | Asp | Leu | Ser | Leu | Gln | Thr | Ser |
| | | | | 80 | | | | | 85 | | | | | 90 |
| Glu | Ile | Lys | Glu | Glu | Glu | Lys | Glu | Leu | Arg | Arg | Thr | Thr | Tyr | Lys |
| | | | | 95 | | | | | 100 | | | | | 105 |
| Leu | Gln | Val | Lys | Asn | Glu | Glu | Val | Lys | Asn | Met | Ser | Leu | Glu | Leu |
| | | | | 110 | | | | | 115 | | | | | 120 |
| Asn | Ser | Lys | Leu | Glu | Ser | Leu | Leu | Glu | Glu | Lys | Ile | Leu | Leu | Gln |
| | | | | 125 | | | | | 130 | | | | | 135 |
| Gln | Lys | Val | Lys | Tyr | Leu | Glu | Glu | Gln | Leu | Thr | Asn | Leu | Ile | Gln |
| | | | | 140 | | | | | 145 | | | | | 150 |
| Asn | Gln | Pro | Glu | Thr | Pro | Glu | His | Pro | Glu | Val | Thr | Ser | Leu | Lys |
| | | | | 155 | | | | | 160 | | | | | 165 |
| Thr | Phe | Val | Glu | Lys | Gln | Asp | Asn | Ser | Ile | Lys | Asp | Leu | Leu | Gln |
| | | | | 170 | | | | | 175 | | | | | 180 |
| Thr | Val | Glu | Asp | Gln | Tyr | Lys | Gln | Leu | Asn | Gln | Gln | His | Ser | Gln |
| | | | | 185 | | | | | 190 | | | | | 195 |
| Ile | Lys | Glu | Ile | Glu | Asn | Gln | Leu | Arg | Arg | Thr | Ser | Ile | Gln | Glu |
| | | | | 200 | | | | | 205 | | | | | 210 |
| Pro | Thr | Glu | Ile | Ser | Leu | Ser | Ser | Lys | Pro | Arg | Ala | Pro | Arg | Thr |
| | | | | 215 | | | | | 220 | | | | | 225 |
| Thr | Pro | Phe | Leu | Gln | Leu | Asn | Glu | Ile | Arg | Asn | Val | Lys | His | Asp |
| | | | | 230 | | | | | 235 | | | | | 240 |
| Gly | Ile | Pro | Ala | Glu | Cys | Thr | Thr | Ile | Tyr | Asn | Arg | Gly | Glu | His |
| | | | | 245 | | | | | 250 | | | | | 255 |
| Thr | Ser | Gly | Met | Tyr | Ala | Ile | Arg | Pro | Ser | Asn | Ser | Gln | Val | Phe |
| | | | | 260 | | | | | 265 | | | | | 270 |
| His | Val | Tyr | Cys | Asp | Val | Ile | Ser | Gly | Ser | Pro | Trp | Thr | Leu | Ile |
| | | | | 275 | | | | | 280 | | | | | 285 |

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln | His | Arg | Ile | Asp | Gly | Ser | Gln | Asn | Phe | Asn | Glu | Thr | Trp | Glu | 290 | 295 | 300 |
| Asn | Tyr | Lys | Tyr | Gly | Phe | Gly | Arg | Leu | Asp | Gly | Glu | Phe | Trp | Leu | 305 | 310 | 315 |
| Gly | Leu | Glu | Lys | Ile | Tyr | Ser | Ile | Val | Lys | Gln | Ser | Asn | Tyr | Val | 320 | 325 | 330 |
| Leu | Arg | Ile | Glu | Leu | Glu | Asp | Trp | Lys | Asp | Asn | Lys | His | Tyr | Ile | 335 | 340 | 345 |
| Glu | Tyr | Ser | Phe | Tyr | Leu | Gly | Asn | His | Glu | Thr | Asn | Tyr | Thr | Leu | 350 | 355 | 360 |
| His | Leu | Val | Ala | Ile | Thr | Gly | Asn | Val | Pro | Asn | Ala | Ile | Pro | Glu | 365 | 370 | 375 |
| Asn | Lys | Asp | Leu | Val | Phe | Ser | Thr | Trp | Asp | His | Lys | Ala | Lys | Gly | 380 | 385 | 390 |
| His | Phe | Asn | Cys | Pro | Glu | Gly | Tyr | Ser | Gly | Gly | Trp | Trp | Trp | His | 395 | 400 | 405 |
| Asp | Glu | Cys | Gly | Glu | Asn | Asn | Leu | Asn | Gly | Lys | Tyr | Asn | Lys | Pro | 410 | 415 | 420 |
| Arg | Ala | Lys | Ser | Lys | Pro | Glu | Arg | Arg | Arg | Gly | Leu | Ser | Trp | Lys | 425 | 430 | 435 |
| Ser | Gln | Asn | Gly | Arg | Leu | Tyr | Ser | Ile | Lys | Ser | Thr | Lys | Met | Leu | 440 | 445 | 450 |
| Ile | His | Pro | Thr | Asp | Ser | Glu | Ser | Phe | Glu | | | | | | 455 | 460 | |

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TGACCAGAAC CTGAAGGTGC TGCCACCCCC TCTGCCCACT ATGCCCACTC 1300
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 CTCAAGAAAG TGGTGATGAT GATCCGACCG AACCCCAACA CCTTCCACTA 2000
 AGCCAGCTCC CCCTCCTGAC CTCTCGTGGC CATTGCCAGG AGCCCACCCT 2050
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 GATGATGGAA CTGAATCGAT ACGGTGTTTT CTGTCCCTCC TACTTTCTTT 2200
 CACACCAGAC AGCCCCCTCAT GTCTCCAGGA CAGGACAGGA CTACAGACAA 2250
 CTCTTTCTTT AAATAAATTA AGTCTCTACA ATAAAAAAA 2290

| | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| Met | Arg | Pro | Leu | Cys | Val | Thr | Cys | Trp | Trp | Leu | Gly | Leu | Leu | Ala | 1 | 5 | 10 | 15 |
| Ala | Met | Gly | Ala | Val | Ala | Gly | Gln | Glu | Asp | Gly | Phe | Glu | Gly | Thr | 20 | 25 | 30 | |
| Glu | Glu | Gly | Ser | Pro | Arg | Glu | Phe | Ile | Tyr | Leu | Asn | Arg | Tyr | Lys | 35 | 40 | 45 | |
| Arg | Ala | Gly | Glu | Ser | Gln | Asp | Lys | Cys | Thr | Tyr | Thr | Phe | Ile | Val | 50 | 55 | 60 | |
| Pro | Gln | Gln | Arg | Val | Thr | Gly | Ala | Ile | Cys | Val | Asn | Ser | Lys | Glu | 65 | 70 | 75 | |
| Pro | Glu | Val | Leu | Leu | Glu | Asn | Arg | Val | His | Lys | Gln | Glu | Leu | Glu | 80 | 85 | 90 | |
| Leu | Leu | Asn | Asn | Glu | Leu | Leu | Lys | Gln | Lys | Arg | Gln | Ile | Glu | Thr | 95 | 100 | 105 | |
| Leu | Gln | Gln | Leu | Val | Glu | Val | Asp | Gly | Gly | Ile | Val | Ser | Glu | Val | 110 | 115 | 120 | |
| Lys | Leu | Leu | Arg | Lys | Glu | Ser | Arg | Asn | Met | Asn | Ser | Arg | Val | Thr | 125 | 130 | 135 | |
| Gln | Leu | Tyr | Met | Gln | Leu | Leu | His | Glu | Ile | Ile | Arg | Lys | Arg | Asp | 140 | 145 | 150 | |
| Asn | Ala | Leu | Glu | Leu | Ser | Gln | Leu | Glu | Asn | Arg | Ile | Leu | Asn | Gln | 155 | 160 | 165 | |
| Thr | Ala | Asp | Met | Leu | Gln | Leu | Ala | Ser | Lys | Tyr | Lys | Asp | Leu | Glu | 170 | 175 | 180 | |
| His | Lys | Tyr | Gln | His | Leu | Ala | Thr | Leu | Ala | His | Asn | Gln | Ser | Glu | 185 | 190 | 195 | |
| Ile | Ile | Ala | Gln | Leu | Glu | Glu | His | Cys | Gln | Arg | Val | Pro | Ser | Ala | 200 | 205 | 210 | |
| Arg | Pro | Val | Pro | Gln | Pro | Pro | Pro | Ala | Ala | Pro | Pro | Arg | Val | Tyr | 215 | 220 | 225 | |
| Gln | Pro | Pro | Thr | Tyr | Asn | Arg | Ile | Ile | Asn | Gln | Ile | Ser | Thr | Asn | 230 | 235 | 240 | |
| Glu | Ile | Gln | Ser | Asp | Gln | Asn | Leu | Lys | Val | Leu | Pro | Pro | Pro | Leu | 245 | 250 | 255 | |
| Pro | Thr | Met | Pro | Thr | Leu | Thr | Ser | Leu | Pro | Ser | Ser | Thr | Asp | Lys | 260 | 265 | 270 | |
| Pro | Ser | Gly | Pro | Trp | Arg | Asp | Cys | Leu | Gln | Ala | Leu | Glu | Asp | Gly | 275 | 280 | 285 | |

Figure 3

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| His | Asp | Thr | Ser | Ser | Ile | Tyr | Leu | Val | Lys | Pro | Glu | Asn | Thr | Asn | 290 | 295 | 300 |
| Arg | Leu | Met | Gln | Val | Trp | Cys | Asp | Gln | Arg | His | Asp | Pro | Gly | Gly | 305 | 310 | 315 |
| Trp | Thr | Val | Ile | Gln | Arg | Arg | Leu | Asp | Gly | Ser | Val | Asn | Phe | Phe | 320 | 325 | 330 |
| Arg | Asn | Trp | Glu | Thr | Tyr | Lys | Gln | Gly | Phe | Gly | Asn | Ile | Asp | Gly | 335 | 340 | 345 |
| Glu | Tyr | Trp | Leu | Gly | Leu | Glu | Asn | Ile | Tyr | Trp | Leu | Thr | Asn | Gln | 350 | 355 | 360 |
| Gly | Asn | Tyr | Lys | Leu | Leu | Val | Thr | Met | Glu | Asp | Trp | Ser | Gly | Arg | 365 | 370 | 375 |
| Lys | Val | Phe | Ala | Glu | Tyr | Ala | Ser | Phe | Arg | Leu | Glu | Pro | Glu | Ser | 380 | 385 | 390 |
| Glu | Tyr | Tyr | Lys | Leu | Arg | Leu | Gly | Arg | Tyr | His | Gly | Asn | Ala | Gly | 395 | 400 | 405 |
| Asp | Ser | Phe | Thr | Trp | His | Asn | Gly | Lys | Gln | Phe | Thr | Thr | Leu | Asp | 410 | 415 | 420 |
| Arg | Asp | His | Asp | Val | Tyr | Thr | Gly | Asn | Cys | Ala | His | Tyr | Gln | Lys | 425 | 430 | 435 |
| Gly | Gly | Trp | Trp | Tyr | Asn | Ala | Cys | Ala | His | Ser | Asn | Leu | Asn | Gly | 440 | 445 | 450 |
| Val | Trp | Tyr | Arg | Gly | Gly | His | Tyr | Arg | Ser | Arg | Tyr | Gln | Asp | Gly | 455 | 460 | 465 |
| Val | Tyr | Trp | Ala | Glu | Phe | Arg | Gly | Gly | Ser | Tyr | Ser | Leu | Lys | Lys | 470 | 475 | 480 |
| Val | Val | Met | Met | Ile | Arg | Pro | Asn | Pro | Asn | Thr | Phe | His | | | 485 | 490 | 493 |

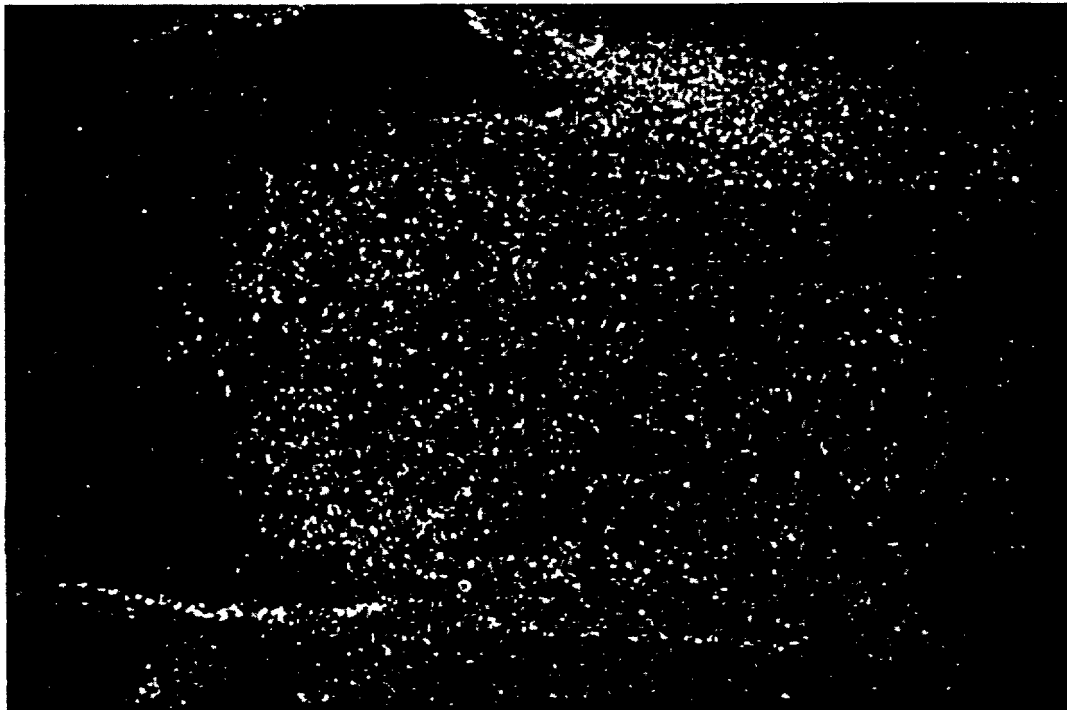
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 AAAAATGCAG CTGCCATTAA AGTCTCAGAT GAACAAACTT CTACACTGAT 250
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 GAAGACTTTTT ACCTGGACCC TAGGTGTGCT ATTCTTCCTA CTAGTGGACA 500
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 CTCCAGAAAT GCATTTTATG TAGTTCTAAA ATCAGCAAAA TATTGGTATT 2700
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 TTTATTATTA CTTTTTTTCT AATTTACTGA TCTTACTACT ACAAAGAAAA 2800
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 AAGTATTAGT GCATGCTCAG AACAGGTGGG ACTAAAACAA ACTCAAGGAA 2900

| | | | |
|---|-----|---------|-----|
| Ala Gly His Ser Val Ser Gly Ile Tyr Met Ile Lys Pro Glu Asn | 290 | 295 | 300 |
| Ser Asn Gly Pro Met Gln Leu Trp Cys Glu Asn Ser Leu Asp Pro | 305 | 310 | 315 |
| Gly Gly Trp Thr Val Ile Gln Lys Arg Thr Asp Gly Ser Val Asn | 320 | 325 | 330 |
| Phe Phe Arg Asn Trp Glu Asn Tyr Lys Lys Gly Phe Gly Asn Ile | 335 | 340 | 345 |
| Asp Gly Glu Tyr Trp Leu Gly Leu Glu Asn Ile Tyr Met Leu Ser | 350 | 355 | 360 |
| Asn Gln Asp Asn Tyr Lys Leu Leu Ile Glu Leu Glu Asp Trp Ser | 365 | 370 | 375 |
| Asp Lys Lys Val Tyr Ala Glu Tyr Ser Ser Phe Arg Leu Glu Pro | 380 | 385 | 390 |
| Glu Ser Glu Phe Tyr Arg Leu Arg Leu Gly Thr Tyr Gln Gly Asn | 395 | 400 | 405 |
| Ala Gly Asp Ser Met Met Trp His Asn Gly Lys Gln Phe Thr Thr | 410 | 415 | 420 |
| Leu Asp Arg Asp Lys Asp Met Tyr Ala Gly Asn Cys Ala His Phe | 425 | 430 | 435 |
| His Lys Gly Gly Trp Trp Tyr Asn Ala Cys Ala His Ser Asn Leu | 440 | 445 | 450 |
| Asn Gly Val Trp Tyr Arg Gly Gly His Tyr Arg Ser Lys His Gln | 455 | 460 | 465 |
| Asp Gly Ile Phe Trp Ala Glu Tyr Arg Gly Gly Ser Tyr Ser Leu | 470 | 475 | 480 |
| Arg Ala Val Gln Met Met Ile Lys Pro Ile Asp | 485 | 490 491 | |

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CTGTACCAGC GGGGAGGCTG GTGGTACCAT GCCTGTGCCC ACTCCAACCT 1400
CAACGGTGTG TGGCACCACG GCGGCCACTA CCGAAGCCGC TACCAGGATG 1450

| 1990 | | 1991 | | 1992 | | 1993 | | 1994 | | 1995 | | 1996 | | 1997 | | 1998 | | 1999 | | 2000 | | 2001 | | 2002 | | 2003 | | 2004 | | 2005 | | 2006 | | 2007 | | 2008 | | 2009 | | 2010 | | 2011 | | 2012 | | 2013 | | 2014 | | 2015 | | 2016 | | 2017 | | 2018 | | 2019 | | 2020 | | 2021 | | 2022 | | 2023 | | 2024 | | 2025 | | 2026 | | 2027 | | 2028 | | 2029 | | 2030 | | 2031 | | 2032 | | 2033 | | 2034 | | 2035 | | 2036 | | 2037 | | 2038 | | 2039 | | 2040 | | 2041 | | 2042 | | 2043 | | 2044 | | 2045 | | 2046 | | 2047 | | 2048 | | 2049 | | 2050 | | 2051 | | 2052 | | 2053 | | 2054 | | 2055 | | 2056 | | 2057 | | 2058 | | 2059 | | 2060 | | 2061 | | 2062 | | 2063 | | 2064 | | 2065 | | 2066 | | 2067 | | 2068 | | 2069 | | 2070 | | 2071 | | 2072 | | 2073 | | 2074 | | 2075 | | 2076 | | 2077 | | 2078 | | 2079 | | 2080 | | 2081 | | 2082 | | 2083 | | 2084 | | 2085 | | 2086 | | 2087 | | 2088 | | 2089 | | 2090 | | 2091 | | 2092 | | 2093 | | 2094 | | 2095 | | 2096 | | 2097 | | 2098 | | 2099 | | 2100 | |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|
| 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 | 2024 | 2025 | 2026 | 2027 | 2028 | 2029 | 2030 | 2031 | 2032 | 2033 | 2034 | 2035 | 2036 | 2037 | 2038 | 2039 | 2040 | 2041 | 2042 | 2043 | 2044 | 2045 | 2046 | 2047 | 2048 | 2049 | 2050 | 2051 | 2052 | 2053 | 2054 | 2055 | 2056 | 2057 | 2058 | 2059 | 2060 | 2061 | 2062 | 2063 | 2064 | 2065 | 2066 | 2067 | 2068 | 2069 | 2070 | 2071 | 2072 | 2073 | 2074 | 2075 | 2076 | 2077 | 2078 | 2079 | 2080 | 2081 | 2082 | 2083 | 2084 | 2085 | 2086 | 2087 | 2088 | 2089 | 2090 | 2091 | 2092 | 2093 | 2094 | 2095 | 2096 | 2097 | 2098 | 2099 | 2100 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |



The white spots represent the silver grains.

Figure 8-A

DNA 22779 H&E

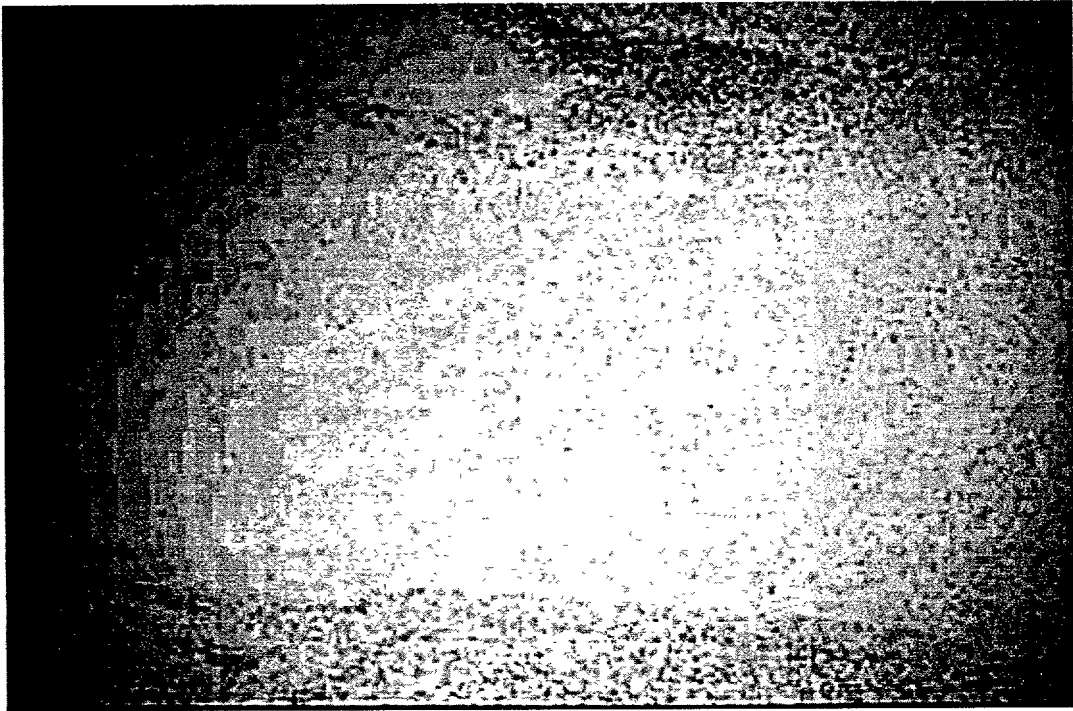


Figure 8-B

00000001.001007

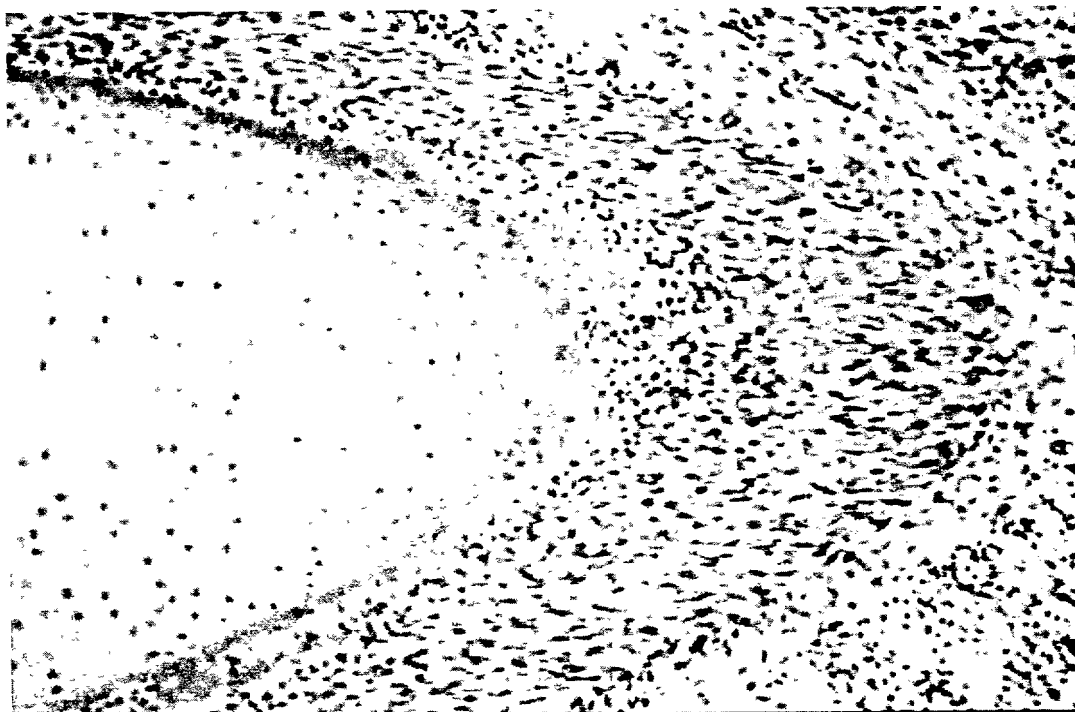
[illegible]

Figure 9A

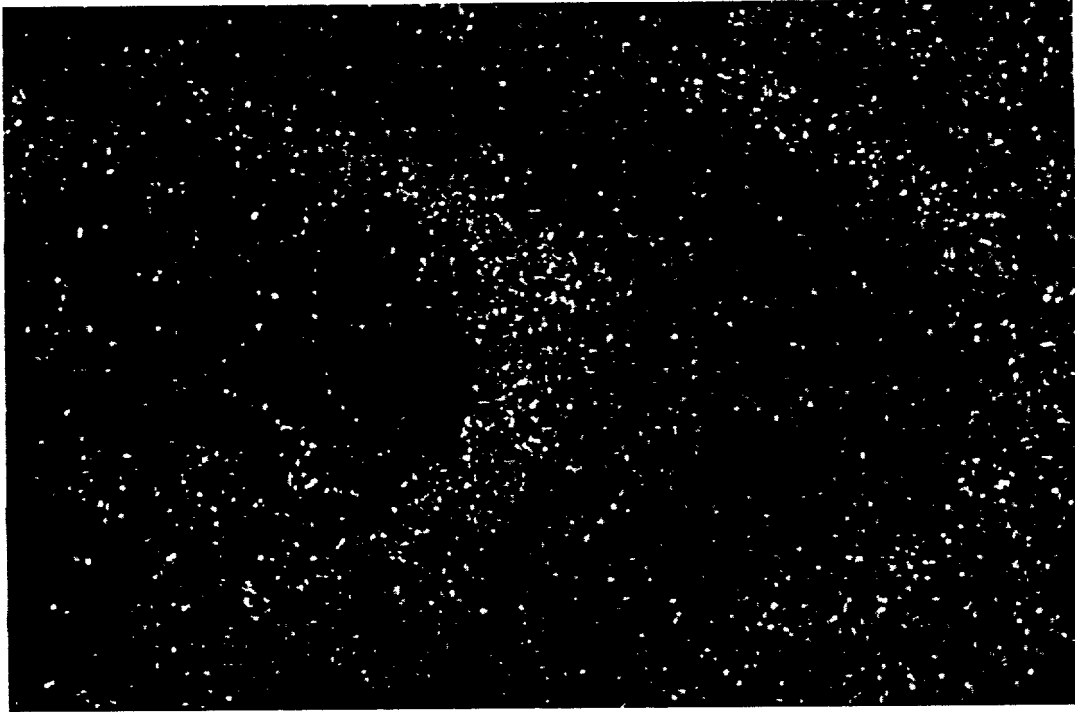
[illegible]

Figure 9B

DNA 23339 H&E



Figure 10-A

00000000.001007

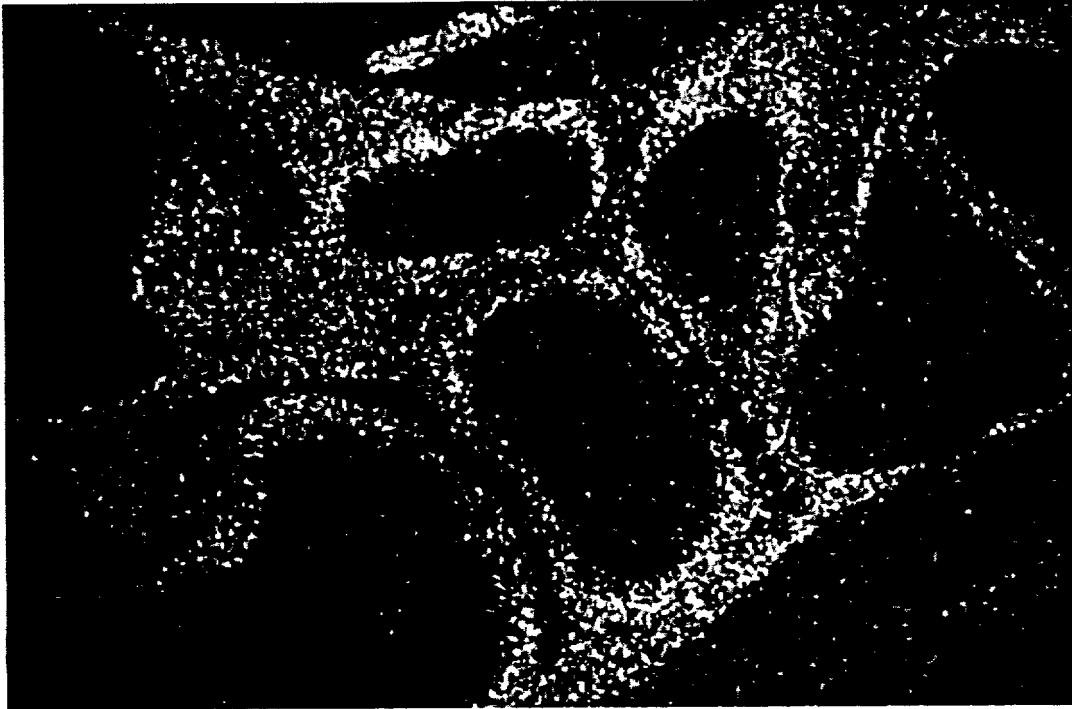
[illegible]

Figure 10-B

NL1 Northern

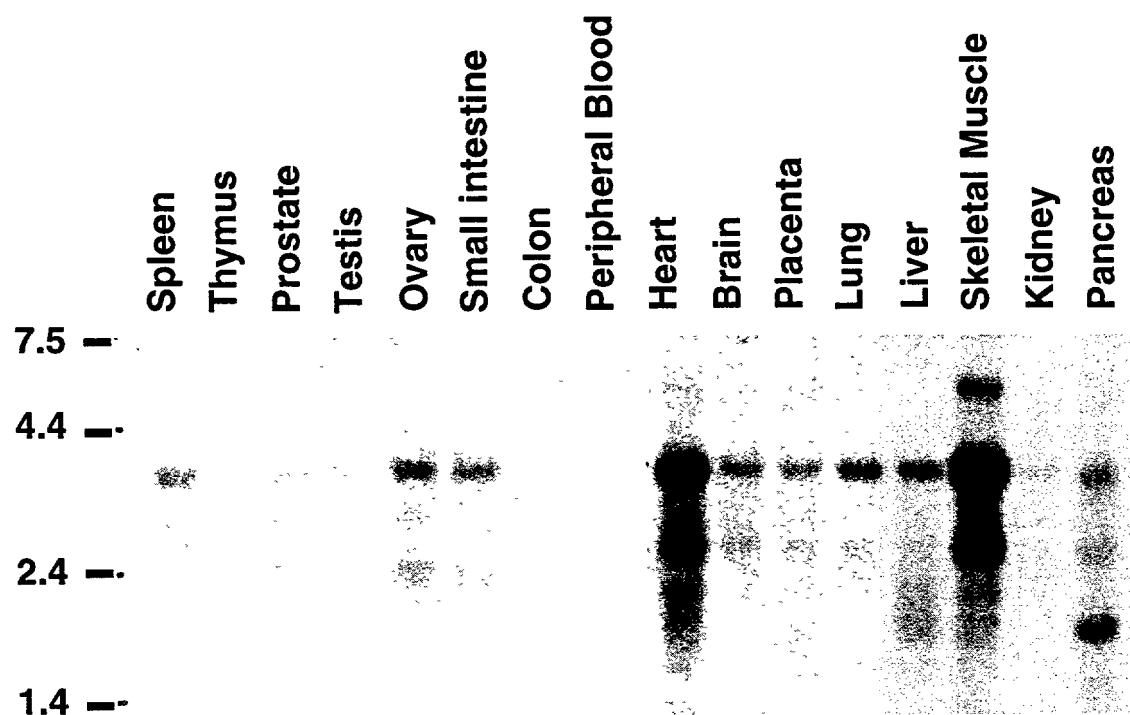


Figure 11

NL5 Northern

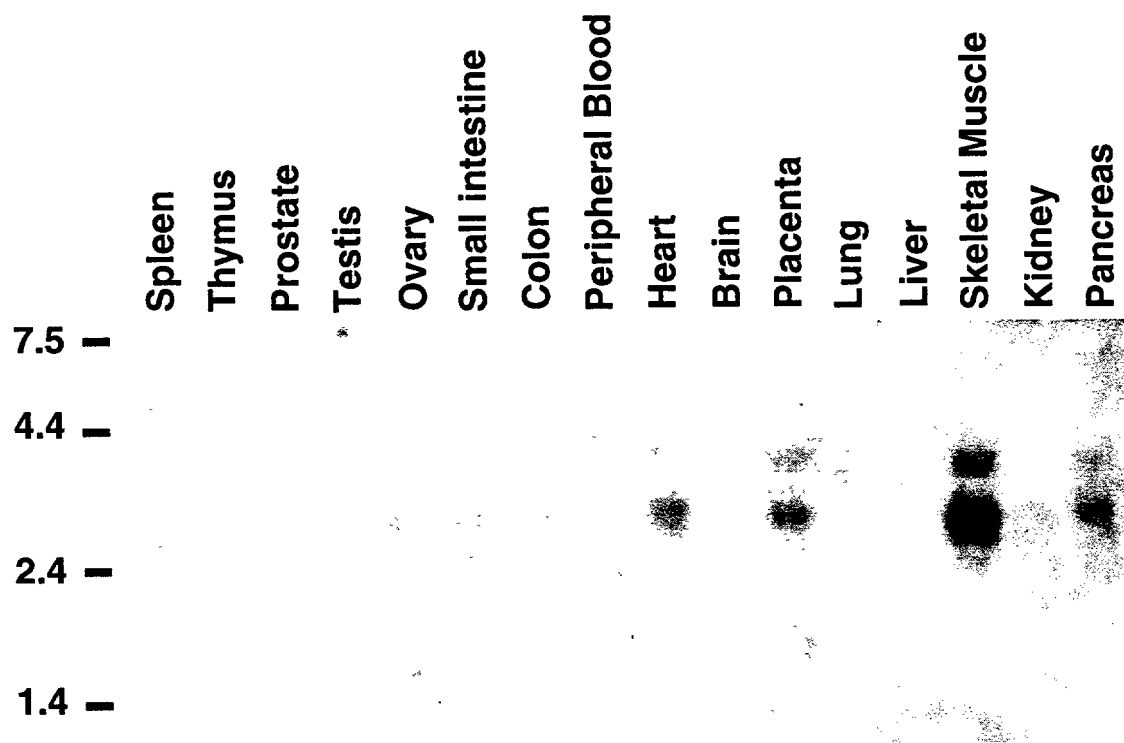


Figure 12

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Godowski, Paul J.
Gurney, Austin L.
- (ii) TITLE OF INVENTION: Tie Ligands
- (iii) NUMBER OF SEQUENCES: 17
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Genentech, Inc.
 - (B) STREET: 1 DNA Way
 - (C) CITY: South San Francisco
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Dreger, Ginger R.
 - (B) REGISTRATION NUMBER: 33,055
 - (C) REFERENCE/DOCKET NUMBER: P1130
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 650/225-3216
 - (B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2290 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCTGAGGGG AGGCCCCGAG CCTTTCTGGG GCCTGGGGGA TCCTCTTGCA 50
CTGGTGGGTG GAGAGAAGCG CCTGCAGCCA ACCAGGGTCA GGCTGTGCTC 100
ACAGTTTCCT CTGGCGGCAT GTAAAGGCTC CACAAAGGAG TTGGGAGTTC 150
AAATGAGGCT GCTGCGGACG GCCTGAGGAT GGACCCCAAG CCCTGGACCT 200
GCCGAGCGTG GCACTGAGGC AGCGGCTGAC GCTACTGTGA GGGAAAGAAG 250
GTTGTGAGCA GCCCCGCAGG ACCCCTGGCC AGCCCTGGCC CCAGCCTCTG 300

CCACTCCAAC CTCAACGGGG TCTGGTACCG CGGGGGCCAT TACCGGAGCC 1900
 GCTACCAGGA CGGAGTCTAC TGGGCTGAGT TCCGAGGAGG CTCTTACTCA 1950
 CTCAAGAAAG TGGTGATGAT GATCCGACCG AACCCCAACA CCTTCCACTA 2000
 AGCCAGCTCC CCCTCCTGAC CTCTCGTGGC CATTGCCAGG AGCCCACCCT 2050
 GGTACAGCTG GCCACAGCAC AAAGAACAAC TCCTCACCAG TTCATCCTGA 2100
 GGCTGGGAGG ACCGGGATGC TGGATTCTGT TTTCCGAAGT CACTGCAGCG 2150
 GATGATGGAA CTGAATCGAT ACGGTGTTTT CTGTCCCTCC TACTTTCCTT 2200
 CACACCAGAC AGCCCCTCAT GTCTCCAGGA CAGGACAGGA CTACAGACAA 2250
 CTCTTTCCTT AAATAAATTA AGTCTCTACA ATAAAAAAAA 2290

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 493 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| Met | Arg | Pro | Leu | Cys | Val | Thr | Cys | Trp | Trp | Leu | Gly | Leu | Leu | Ala | 1 | 5 | 10 | 15 |
| Ala | Met | Gly | Ala | Val | Ala | Gly | Gln | Glu | Asp | Gly | Phe | Glu | Gly | Thr | 20 | 25 | 30 | |
| Glu | Glu | Gly | Ser | Pro | Arg | Glu | Phe | Ile | Tyr | Leu | Asn | Arg | Tyr | Lys | 35 | 40 | 45 | |
| Arg | Ala | Gly | Glu | Ser | Gln | Asp | Lys | Cys | Thr | Tyr | Thr | Phe | Ile | Val | 50 | 55 | 60 | |
| Pro | Gln | Gln | Arg | Val | Thr | Gly | Ala | Ile | Cys | Val | Asn | Ser | Lys | Glu | 65 | 70 | 75 | |
| Pro | Glu | Val | Leu | Leu | Glu | Asn | Arg | Val | His | Lys | Gln | Glu | Leu | Glu | 80 | 85 | 90 | |
| Leu | Leu | Asn | Asn | Glu | Leu | Leu | Lys | Gln | Lys | Arg | Gln | Ile | Glu | Thr | 95 | 100 | 105 | |
| Leu | Gln | Gln | Leu | Val | Glu | Val | Asp | Gly | Gly | Ile | Val | Ser | Glu | Val | 110 | 115 | 120 | |
| Lys | Leu | Leu | Arg | Lys | Glu | Ser | Arg | Asn | Met | Asn | Ser | Arg | Val | Thr | 125 | 130 | 135 | |
| Gln | Leu | Tyr | Met | Gln | Leu | Leu | His | Glu | Ile | Ile | Arg | Lys | Arg | Asp | 140 | 145 | 150 | |
| Asn | Ala | Leu | Glu | Leu | Ser | Gln | Leu | Glu | Asn | Arg | Ile | Leu | Asn | Gln | 155 | 160 | 165 | |
| Thr | Ala | Asp | Met | Leu | Gln | Leu | Ala | Ser | Lys | Tyr | Lys | Asp | Leu | Glu | | | | |

ACCACCTGAT CTGGCAACTT CTCCCACCAA AAGCCCTTTC AAGATACCAC 1250
 CGGTAAC TTT CATCAATGAA GGACCATTCA AAGACTGTCA GCAAGCAAAA 1300
 GAAGCTGGGC ATTCGGTCAG TGGGATTTAT ATGATTAAAC CTGAAAACAG 1350
 CAATGGACCA ATGCAGTTAT GGTGTGAAAA CAGTTTGGAC CCTGGGGGTT 1400
 GGACTGTTAT TCAGAAAAGA ACAGACGGCT CTGTCAACTT CTTTCAGAAAT 1450
 TGGGAAAATT ATAAGAAAGG GTTTGGAAAC ATTGACGGAG AATACTGGCT 1500
 TGGACTGGAA AATATCTATA TGCTTAGCAA TCAAGATAAT TACAAGTTAT 1550
 TGATTGAATT AGAAGACTGG AGTGATAAAA AAGTCTATGC AGAATACAGC 1600
 AGCTTTCGTC TGGAACCTGA AAGTGAATTC TATAGACTGC GCCTGGGAAC 1650
 TTACCAGGGA AATGCAGGGG ATTCTATGAT GTGGCATAAT GGTAAACAAT 1700
 TCACCACACT GGACAGAGAT AAAGATATGT ATGCAGGAAA CTGCGCCAC 1750
 TTTTCATAAAG GAGGCTGGTG GTACAATGCC TGTGCACATT CTAACCTAAA 1800
 TGGAGTATGG TACAGAGGAG GCCATTACAG AAGCAAGCAC CAAGATGGAA 1850
 TTTTCTGGGC CGAATACAGA GCGGGTCAT ACTCCTTAAG AGCAGTTCAG 1900
 ATGATGATCA AGCCTATTGA CTGAAGAGAG AACTCGCCA ATTTAAATGA 1950
 CACAGAACTT TGTACTTTTC AGCTCTTAAA AATGTAAATG TTACATGTAT 2000
 ATTACTTGGC ACAATTTATT TCTACACAGA AAGTTTTTAA AATGAATTTT 2050
 ACCGTAAC TA AAAAGGGAA CCTATAAATG TAGTTTCATC TGTCGTCAAT 2100
 TACTGCAGAA AATTATGTGT ATCCACAACC TAGTTATTTT AAAAATTATG 2150
 TTGACTAAAT ACAAAGTTTG TTTTCTAAAA TGTAATATT TGCCACAATG 2200
 TAAAGCAAAT CTTAGCTATA TTTTAAATCA TAAATAACAT GTTCAAGATA 2250
 CTTAACAATT TATTTAAAT CTAAGATTGC TCTAACGTCT AGTGAAAAAA 2300
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 GATTAAATCT GAATTCTTAA TAATATATCC TATGCTGATT TTCCCAAAC 2500
 ATGACCATA GTATTAAATA CATATCATTT TTAAAAATAA AAAAAACCC 2550
 AAAAATAATG CATGCATAAT TTAAATGGTC AATTTATAAA GACAAATCTA 2600
 TGAATGAATT TTTCAGTGTT ATCTTCATAT GATATGCTGA ACACCAAAT 2650
 CTCCAGAAAT GCATTTTATG TAGTTCTAAA ATCAGCAAAA TATTGGTATT 2700
 AAAAAATGC AGAATATTTA GTGTGCTACA GATCTGAATT ATAGTTCTAA 2750

TTTATTATTA CTTTTTTTCT AATTTACTGA TCTTACTACT ACAAAGAAAA 2800
 AAAAACCCAA CCCATCTGCA ATTCAAATCA GAAAGTTTGG ACAGCTTTAC 2850
 AAGTATTAGT GCATGCTCAG AACAGGTGGG ACTAAAACAA ACTCAAGGAA 2900
 CTGTTGGCTG TTTTCCCGAT ACTGAGAATT CAACAGCTCC AGAGCAGAAG 2950
 CCACAGGGGC ATAGCTTAGT CCAAAGTGCT AATTTCAATT TACAGTGTAT 3000
 GTAACGCTTA GTCTCACAGT GTCTTTAACT CATCTTTGCA ATCAACAAC 3050
 TTACTAGTGA CTTTCTGGAA CAATTTCTTT TCAGGAATAC ATATTCAC 3100
 CTTAGAGGTG ACCTTGCCCTT AATATATTTG TGAAGTTAAA ATTTTAAAGA 3150
 TAGCTCATGA AACTTTTGCT TAAGCAAAAA GAAAACCTCG AATTGAAATG 3200
 TGTGAGGCAA ACTATGCATG GGAATAGCTT AATGTGAAGA TAATCATTTG 3250
 GACAACTCAA ATCCATCAAC ATGACCAATG TTTTTCATCT GCCACATCTC 3300
 AAAATAAAAC TTCTGGTGAA ACAAATTAAC CAAAATATCC AAACCTCAAA 3350
 AAAAA 3355

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 491 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

| | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| Met | Lys | Thr | Phe | Thr | Trp | Thr | Leu | Gly | Val | Leu | Phe | Phe | Leu | Leu | 1 | 5 | 10 | 15 |
| Val | Asp | Thr | Gly | His | Cys | Arg | Gly | Gly | Gln | Phe | Lys | Ile | Lys | Lys | 20 | 25 | 30 | |
| Ile | Asn | Gln | Arg | Arg | Tyr | Pro | Arg | Ala | Thr | Asp | Gly | Lys | Glu | Glu | 35 | 40 | 45 | |
| Ala | Lys | Lys | Cys | Ala | Tyr | Thr | Phe | Leu | Val | Pro | Glu | Gln | Arg | Ile | 50 | 55 | 60 | |
| Thr | Gly | Pro | Ile | Cys | Val | Asn | Thr | Lys | Gly | Gln | Asp | Ala | Ser | Thr | 65 | 70 | 75 | |
| Ile | Lys | Asp | Met | Ile | Thr | Arg | Met | Asp | Leu | Glu | Asn | Leu | Lys | Asp | 80 | 85 | 90 | |
| Val | Leu | Ser | Arg | Gln | Lys | Arg | Glu | Ile | Asp | Val | Leu | Gln | Leu | Val | 95 | 100 | 105 | |
| Val | Asp | Val | Asp | Gly | Asn | Ile | Val | Asn | Glu | Val | Lys | Leu | Leu | Arg | 110 | 115 | 120 | |
| Lys | Glu | Ser | Arg | Asn | Met | Asn | Ser | Arg | Val | Thr | Gln | Leu | Tyr | Met | 125 | 130 | 135 | |

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln | Leu | Leu | His | Glu | Ile | Ile | Arg | Lys | Arg | Asp | Asn | Ser | Leu | Glu |
| | | | | 140 | | | | | 145 | | | | | 150 |
| Leu | Ser | Gln | Leu | Glu | Asn | Lys | Ile | Leu | Asn | Val | Thr | Thr | Glu | Met |
| | | | | 155 | | | | | 160 | | | | | 165 |
| Leu | Lys | Met | Ala | Thr | Arg | Tyr | Arg | Glu | Leu | Glu | Val | Lys | Tyr | Ala |
| | | | | 170 | | | | | 175 | | | | | 180 |
| Ser | Leu | Thr | Asp | Leu | Val | Asn | Asn | Gln | Ser | Val | Met | Ile | Thr | Leu |
| | | | | 185 | | | | | 190 | | | | | 195 |
| Leu | Glu | Glu | Gln | Cys | Leu | Arg | Ile | Phe | Ser | Arg | Gln | Asp | Thr | His |
| | | | | 200 | | | | | 205 | | | | | 210 |
| Val | Ser | Pro | Pro | Leu | Val | Gln | Val | Val | Pro | Gln | His | Ile | Pro | Asn |
| | | | | 215 | | | | | 220 | | | | | 225 |
| Ser | Gln | Gln | Tyr | Thr | Pro | Gly | Leu | Leu | Gly | Gly | Asn | Glu | Ile | Gln |
| | | | | 230 | | | | | 235 | | | | | 240 |
| Arg | Asp | Pro | Gly | Tyr | Pro | Arg | Asp | Leu | Met | Pro | Pro | Pro | Asp | Leu |
| | | | | 245 | | | | | 250 | | | | | 255 |
| Ala | Thr | Ser | Pro | Thr | Lys | Ser | Pro | Phe | Lys | Ile | Pro | Pro | Val | Thr |
| | | | | 260 | | | | | 265 | | | | | 270 |
| Phe | Ile | Asn | Glu | Gly | Pro | Phe | Lys | Asp | Cys | Gln | Gln | Ala | Lys | Glu |
| | | | | 275 | | | | | 280 | | | | | 285 |
| Ala | Gly | His | Ser | Val | Ser | Gly | Ile | Tyr | Met | Ile | Lys | Pro | Glu | Asn |
| | | | | 290 | | | | | 295 | | | | | 300 |
| Ser | Asn | Gly | Pro | Met | Gln | Leu | Trp | Cys | Glu | Asn | Ser | Leu | Asp | Pro |
| | | | | 305 | | | | | 310 | | | | | 315 |
| Gly | Gly | Trp | Thr | Val | Ile | Gln | Lys | Arg | Thr | Asp | Gly | Ser | Val | Asn |
| | | | | 320 | | | | | 325 | | | | | 330 |
| Phe | Phe | Arg | Asn | Trp | Glu | Asn | Tyr | Lys | Lys | Gly | Phe | Gly | Asn | Ile |
| | | | | 335 | | | | | 340 | | | | | 345 |
| Asp | Gly | Glu | Tyr | Trp | Leu | Gly | Leu | Glu | Asn | Ile | Tyr | Met | Leu | Ser |
| | | | | 350 | | | | | 355 | | | | | 360 |
| Asn | Gln | Asp | Asn | Tyr | Lys | Leu | Leu | Ile | Glu | Leu | Glu | Asp | Trp | Ser |
| | | | | 365 | | | | | 370 | | | | | 375 |
| Asp | Lys | Lys | Val | Tyr | Ala | Glu | Tyr | Ser | Ser | Phe | Arg | Leu | Glu | Pro |
| | | | | 380 | | | | | 385 | | | | | 390 |
| Glu | Ser | Glu | Phe | Tyr | Arg | Leu | Arg | Leu | Gly | Thr | Tyr | Gln | Gly | Asn |
| | | | | 395 | | | | | 400 | | | | | 405 |
| Ala | Gly | Asp | Ser | Met | Met | Trp | His | Asn | Gly | Lys | Gln | Phe | Thr | Thr |
| | | | | 410 | | | | | 415 | | | | | 420 |
| Leu | Asp | Arg | Asp | Lys | Asp | Met | Tyr | Ala | Gly | Asn | Cys | Ala | His | Phe |
| | | | | 425 | | | | | 430 | | | | | 435 |
| His | Lys | Gly | Gly | Trp | Trp | Tyr | Asn | Ala | Cys | Ala | His | Ser | Asn | Leu |

00033021.051597

| | | |
|-------------------------------------|---------------------|-----|
| 440 | 445 | 450 |
| Asn Gly Val Trp Tyr Arg Gly Gly His | Tyr Arg Ser Lys His | Gln |
| 455 | 460 | 465 |
| Asp Gly Ile Phe Trp Ala Glu Tyr Arg | Gly Gly Ser Tyr Ser | Leu |
| 470 | 475 | 480 |
| Arg Ala Val Gln Met Met Ile Lys Pro | Ile Asp | |
| 485 | 490 491 | |

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1780 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

GGCTCAGAGG CCCCACTGGA CCCTCGGCTC TTCCTTGGAC TTCTTGTGTG 50
TTCTGTGAGC TTCGCTGGAT TCAGGGTCTT GGGCATCAGA GGTGAGAGGG 100
TGGAAGGTC CGCCGCGATG GGAAGCCCT GGCTGCGTGC GCTACAGCTG 150
CTGCTCCTGC TGGGCGCGTC GTGGGCGCGG GCGGGCGCCC CGCGCTGCAC 200
CTACACCTTC GTGCTGCCCC CGCAGAAGTT CACGGGCGCT GTGTGCTGGA 250
GCGGCCCCGC ATCCACGCGG GCGACGCCG AGGCCGCCAA CGCCAGCGAG 300
CTGGCGGCGC TGCGCATGCG CGTCGGCCGC CACGAGGAGC TGTTACGCGA 350
GCTGCAGAGG CTGGCGGCGG CCGACGGCGC CGTGGCCGGC GAGGTGCGCG 400
CGCTGCGCAA GGAGAGCCGC GGCCTGAGCG CGCGCCTGGG CCAGTTGCGC 450
GCGCAGCTGC AGCACGAGGC GGGGCCCCGG GCGGGCCCCG GGGCGGATCT 500
GGGGGCGGAG CCTGCCGCGG CGCTGGCGCT GCTCGGGGAG CGCGTGCTCA 550
ACGCGTCCGC CGAGGCTCAG CGCGCAGCCG CCCGGTTCCA CCAGCTGGAC 600
GTCAAGTTCC GCGAGCTGGC GCAGCTCGTC ACCCAGCAGA GCAGTCTCAT 650
CGCCCGCCTG GAGCGCCTGT GCGGGGAGG CGCGGGCGGG CAGCAGCAGG 700
TCCTGCCGCC ACCCCCACTG GTGCCTGTGG TTCCGGTCCG TCTTGTGGGT 750
AGCACCAGTG ACACCAGTAG GATGCTGGAC CCAGCCCCAG AGCCCCAGAG 800
AGACCAGACC CAGAGACAGC AGGAGCCCAT GGCTTCTCCC ATGCCTGCAG 850
GTCACCCTGC GGTCCCCACC AAGCCTGTGG GCCCGTGGCA GGATTGTGCA 900
GAGGCCCGCC AGGCAGGCCA TGAACAGAGT GGAGTGTATG AACTGCGAGT 950
GGGCCGTAC GTAGTGTACG TATGGTGTGA GCAGCAACTG GAGGGTGGAG 1000

```

GCTGGACTGT GATCCAGCGG AGGCAAGATG GTTCAGTCAA CTTCTTCACT 1050
 ACCTGGCAGC ACTATAAGGC GGGCTTTGGG CGGCCAGACG GAGAATACTG 1100
 GCTGGGCCTT GAACCCGTGT ATCAGCTGAC CAGCCGTGGG GACCATGAGC 1150
 TGCTGGTTCT CCTGGAGGAC TGGGGGGGCC GTGGAGCACG TGCCCACTAT 1200
 GATGGCTTCT CCCTGGAACC CGAGAGCGAC CACTACCGCC TGC GGCTTGG 1250
 CCAGTACCAT GGTGATGCTG GAGACTCTCT TTCTTGGCAC AATGACAAGC 1300
 CCTTCAGCAC CGTGGATAGG GACCGAGACT CCTATTCTGG TAACTGTGCC 1350
 CTGTACCAGC GGGGAGGCTG GTGGTACCAT GCCTGTGCCC ACTCCAACCT 1400
 CAACGGTGTG TGGCACCACG GCGGCCACTA CCGAAGCCGC TACCAGGATG 1450
 GTGTCTACTG GGCTGAGTTT CGTGGTGGGG CATATTCTCT CAGGAAGGCC 1500
 GCCATGCTCA TTCGGCCCTT GAAGCTGTGA CTCTGTGTTC CTCTGTCCCC 1550
 TAGGCCCTAG AGGACATTGG TCAGCAGGAG CCCAAGTTGT TCTGGCCACA 1600
 CCTTCTTTGT GGCTCAGTGC CAATGTGTCC CACAGAACTT CCCACTGTGG 1650
 ATCTGTGACC CTGGGCGCTG AAAATGGGAC CCAGGAATCC CCCCCGTCAA 1700
 TATCTTGGCC TCAGATGGCT CCCCAAGGTC ATTCATATCT CGGTTTGAGC 1750
 TCATATCTTA TAATAACACA AAGTAGCCAC 1780

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 470 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

| | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|
| Met | Gly | Lys | Pro | Trp | Leu | Arg | Ala | Leu | Gln | Leu | Leu | Leu | Leu | Leu | 1 | 5 | 10 | 15 |
| Gly | Ala | Ser | Trp | Ala | Arg | Ala | Gly | Ala | Pro | Arg | Cys | Thr | Tyr | Thr | 20 | 25 | 30 | |
| Phe | Val | Leu | Pro | Pro | Gln | Lys | Phe | Thr | Gly | Ala | Val | Cys | Trp | Ser | 35 | 40 | 45 | |
| Gly | Pro | Ala | Ser | Thr | Arg | Ala | Thr | Pro | Glu | Ala | Ala | Asn | Ala | Ser | 50 | 55 | 60 | |
| Glu | Leu | Ala | Ala | Leu | Arg | Met | Arg | Val | Gly | Arg | His | Glu | Glu | Leu | 65 | 70 | 75 | |
| Leu | Arg | Glu | Leu | Gln | Arg | Leu | Ala | Ala | Ala | Asp | Gly | Ala | Val | Ala | 80 | 85 | 90 | |
| Gly | Glu | Val | Arg | Ala | Leu | Arg | Lys | Glu | Ser | Arg | Gly | Leu | Ser | Ala | 95 | 100 | 105 | |

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 410 | | 415 | | 420 | | | | | | | | | |
| His | Ala | Cys | Ala | His | Ser | Asn | Leu | Asn | Gly | Val | Trp | His | His | Gly |
| | | | | 425 | | | | | 430 | | | | | 435 |
| Gly | His | Tyr | Arg | Ser | Arg | Tyr | Gln | Asp | Gly | Val | Tyr | Trp | Ala | Glu |
| | | | | 440 | | | | | 445 | | | | | 450 |
| Phe | Arg | Gly | Gly | Ala | Tyr | Ser | Leu | Arg | Lys | Ala | Ala | Met | Leu | Ile |
| | | | | 455 | | | | | 460 | | | | | 465 |
| Arg | Pro | Leu | Lys | Leu | | | | | | | | | | |
| | | | | 470 | | | | | | | | | | |

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGACGAAC CAAGGCAACT ACAAACCTCCT GGT 33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCGGCCGGA CCAGTCCTCC ATGGTCACCA GGAGTTTGTA G 41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGGTGAAC TGCTTGCCGT TGTGCCATGT AAA 33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGTTATCC CAGAGATTTA ATGCCACCA 29

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTGGTGGGAG AAGTTGCCAG ATCAGGTGGT GGCA 34

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTCACACCAT AACTGCATTG GTCCA 25

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACGTAGTTCC AGTATGGTGT GAGCAGCAAC TGGA 34

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGTCCAGCCT CCACCCTCCA GTTGCT 26

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCCCAGTCCT CCAGGAGAAC CAGCA 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2042 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCGGACGCGT GGGTGAAATT GAAAATCAAG ATAAAAATGT TCACAATTAA 50
GCTCCTTCTT TTTATTGTTC CTCTAGTTAT TTCCTCCAGA ATTGATCAAG 100
ACAATTCATC ATTTGATTCT CTATCTCCAG AGCCAAAATC AAGATTTGCT 150
ATGTTAGACG ATGTAAAAAT TTTAGCCAAT GGCCCTCCTTC AGTTGGGACA 200
TGGTCTTAAA GACTTTGTCC ATAAGACGAA GGGCCAAATT AATGACATAT 250
TTCAAAAACT CAACATATTT GATCAGTCTT TTTATGATCT ATCGCTGCAA 300
ACCAGTGAAA TCAAAGAAGA AGAAAAGGAA CTGAGAAGAA CTACATATAA 350
ACTACAAGTC AAAAATGAAG AGGTAAAGAA TATGTCACTT GAACTCAACT 400
CAAACTTGA AAGCCTCCTA GAAGAAAAAA TTCTACTTCA ACAAAAAGTG 450
AAATATTTAG AAGAGCAACT AACTAACTTA ATTCAAAATC AACCTGAAAC 500
TCCAGAACAC CCAGAAGTAA CTTCACTTAA AACTTTTGTA GAAAAACAAG 550
ATAATAGCAT CAAAGACCTT CTCCAGACCG TGGAAGACCA ATATAAACAA 600
TTAAACCAAC AGCATAGTCA AATAAAAGAA ATAGAAAATC AGCTCAGAAG 650
GACTAGTATT CAAGAACCCA CAGAAATTTT TCTATCTTCC AAGCCAAGAG 700
CACCAAGAAC TACTCCCTTT CTTCACTTGA ATGAAATAAG AAATGTAAAA 750
CATGATGGCA TTCCTGCTGA ATGTACCACC ATTTATAACA GAGGTGAACA 800
TACAAGTGGC ATGTATGCCA TCAGACCCAG CAACTCTCAA GTTTTTCATG 850
TCTACTGTGA TGTTATATCA GGTAGTCCAT GGACATTAAT TCAACATCGA 900
ATAGATGGAT CAAAAAATT CAATGAAACG TGGGAGAACT ACAAATATGG 950
TTTTTGGGAGG CTTGATGGAG AATTTTGGTT GGGCCTAGAG AAGATATACT 1000
CCATAGTGAA GCAATCTAAT TATGTTTTAC GAATTGAGTT GGAAGACTGG 1050

AAAGACAACA AACATTATAT TGAATATTCT TTTTACTTGG GAAATCACGA 1100
 AACCAACTAT ACGCTACATC TAGTTGCGAT TACTGGCAAT GTCCCCAATG 1150
 CAATCCCGGA AAACAAAGAT TTGGTGT TTTT CTACTTGGGA TCACAAAGCA 1200
 AAAGGACACT TCAACTGTCC AGAGGGTTAT TCAGGAGGCT GGTGGTGGCA 1250
 TGATGAGTGT GGAGAAAACA ACCTAAATGG TAAATATAAC AAACCAAGAG 1300
 CAAAATCTAA GCCAGAGAGG AGAAGAGGAT TATCTTGGAA GTCTCAAAAT 1350
 GGAAGGTAT ACTCTATAAA ATCAACCAA ATGTTGATCC ATCCAACAGA 1400
 TTCAGAAAGC TTTGAATGAA CTGAGGCAAT TTAAAGGCAT ATTTAACCAT 1450
 TAACTCATTC CAAGTTAATG TGGTCTAATA ATCTGGTATA AATCCTTAAG 1500
 AGAAAGCTTG AGAAATAGAT TTTTTTTTATC TTAAAGTCAC TGTCTATTTA 1550
 AGATTAAACA TACAATCACA TAACCTTAAA GAATACCGTT TACATTTCTC 1600
 AATCAAAATT CTTATAATAC TATTTGTTTT AAATTTTGTG ATGTGGGAAT 1650
 CAATTTTAGA TGGTCACAAT CTAGATTATA ATCAATAGGT GAACTTATTA 1700
 AATAACTTTT CTAAATAAAA AATTTAGAGA CTTTTATTTT AAAAGGCATC 1750
 ATATGAGCTA ATATCACAAC TTTCCCAGTT TAAAAACTA GTACTCTTGT 1800
 TAAAACTCTA AACTTGACTA AATACAGAGG ACTGGTAATT GTACAGTTCT 1850
 TAAATGTTGT AGTATTAATT TCAAAACTAA AAATCGTCAG CACAGAGTAT 1900
 GTGTAAAAAT CTGTAATACA AATTTTTTAAA CTGATGCTTC ATTTTGCTAC 1950
 AAAATAATTT GGAGTAAATG TTTGATATGA TTTATTTATG AAACCTAATG 2000
 AAGCAGAATT AAATACTGTA TTAAATAAG TTCGCTGTCT TT 2042

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 460 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Phe | Thr | Ile | Lys | Leu | Leu | Leu | Phe | Ile | Val | Pro | Leu | Val | Ile |
| 1 | | | | 5 | | | | 10 | | | | | | 15 |
| Ser | Ser | Arg | Ile | Asp | Gln | Asp | Asn | Ser | Ser | Phe | Asp | Ser | Leu | Ser |
| | | | | 20 | | | | 25 | | | | | | 30 |
| Pro | Glu | Pro | Lys | Ser | Arg | Phe | Ala | Met | Leu | Asp | Asp | Val | Lys | Ile |
| | | | | 35 | | | | 40 | | | | | | 45 |
| Leu | Ala | Asn | Gly | Leu | Leu | Gln | Leu | Gly | His | Gly | Leu | Lys | Asp | Phe |
| | | | | 50 | | | | 55 | | | | | | 60 |

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Val | His | Lys | Thr | Lys | Gly | Gln | Ile | Asn | Asp | Ile | Phe | Gln | Lys | Leu | 65 | 70 | 75 |
| Asn | Ile | Phe | Asp | Gln | Ser | Phe | Tyr | Asp | Leu | Ser | Leu | Gln | Thr | Ser | 80 | 85 | 90 |
| Glu | Ile | Lys | Glu | Glu | Glu | Lys | Glu | Leu | Arg | Arg | Thr | Thr | Tyr | Lys | 95 | 100 | 105 |
| Leu | Gln | Val | Lys | Asn | Glu | Glu | Val | Lys | Asn | Met | Ser | Leu | Glu | Leu | 110 | 115 | 120 |
| Asn | Ser | Lys | Leu | Glu | Ser | Leu | Leu | Glu | Glu | Lys | Ile | Leu | Leu | Gln | 125 | 130 | 135 |
| Gln | Lys | Val | Lys | Tyr | Leu | Glu | Glu | Gln | Leu | Thr | Asn | Leu | Ile | Gln | 140 | 145 | 150 |
| Asn | Gln | Pro | Glu | Thr | Pro | Glu | His | Pro | Glu | Val | Thr | Ser | Leu | Lys | 155 | 160 | 165 |
| Thr | Phe | Val | Glu | Lys | Gln | Asp | Asn | Ser | Ile | Lys | Asp | Leu | Leu | Gln | 170 | 175 | 180 |
| Thr | Val | Glu | Asp | Gln | Tyr | Lys | Gln | Leu | Asn | Gln | Gln | His | Ser | Gln | 185 | 190 | 195 |
| Ile | Lys | Glu | Ile | Glu | Asn | Gln | Leu | Arg | Arg | Thr | Ser | Ile | Gln | Glu | 200 | 205 | 210 |
| Pro | Thr | Glu | Ile | Ser | Leu | Ser | Ser | Lys | Pro | Arg | Ala | Pro | Arg | Thr | 215 | 220 | 225 |
| Thr | Pro | Phe | Leu | Gln | Leu | Asn | Glu | Ile | Arg | Asn | Val | Lys | His | Asp | 230 | 235 | 240 |
| Gly | Ile | Pro | Ala | Glu | Cys | Thr | Thr | Ile | Tyr | Asn | Arg | Gly | Glu | His | 245 | 250 | 255 |
| Thr | Ser | Gly | Met | Tyr | Ala | Ile | Arg | Pro | Ser | Asn | Ser | Gln | Val | Phe | 260 | 265 | 270 |
| His | Val | Tyr | Cys | Asp | Val | Ile | Ser | Gly | Ser | Pro | Trp | Thr | Leu | Ile | 275 | 280 | 285 |
| Gln | His | Arg | Ile | Asp | Gly | Ser | Gln | Asn | Phe | Asn | Glu | Thr | Trp | Glu | 290 | 295 | 300 |
| Asn | Tyr | Lys | Tyr | Gly | Phe | Gly | Arg | Leu | Asp | Gly | Glu | Phe | Trp | Leu | 305 | 310 | 315 |
| Gly | Leu | Glu | Lys | Ile | Tyr | Ser | Ile | Val | Lys | Gln | Ser | Asn | Tyr | Val | 320 | 325 | 330 |
| Leu | Arg | Ile | Glu | Leu | Glu | Asp | Trp | Lys | Asp | Asn | Lys | His | Tyr | Ile | 335 | 340 | 345 |
| Glu | Tyr | Ser | Phe | Tyr | Leu | Gly | Asn | His | Glu | Thr | Asn | Tyr | Thr | Leu | 350 | 355 | 360 |
| His | Leu | Val | Ala | Ile | Thr | Gly | Asn | Val | Pro | Asn | Ala | Ile | Pro | Glu | | | |

| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| 365 | | | | | | | | | 370 | | | | | 375 | |
| Asn | Lys | Asp | Leu | Val | Phe | Ser | Thr | Trp | Asp | His | Lys | Ala | Lys | Gly | |
| | | | | 380 | | | | | 385 | | | | | 390 | |
| His | Phe | Asn | Cys | Pro | Glu | Gly | Tyr | Ser | Gly | Gly | Trp | Trp | Trp | His | |
| | | | | 395 | | | | | 400 | | | | | 405 | |
| Asp | Glu | Cys | Gly | Glu | Asn | Asn | Leu | Asn | Gly | Lys | Tyr | Asn | Lys | Pro | |
| | | | | 410 | | | | | 415 | | | | | 420 | |
| Arg | Ala | Lys | Ser | Lys | Pro | Glu | Arg | Arg | Arg | Gly | Leu | Ser | Trp | Lys | |
| | | | | 425 | | | | | 430 | | | | | 435 | |
| Ser | Gln | Asn | Gly | Arg | Leu | Tyr | Ser | Ile | Lys | Ser | Thr | Lys | Met | Leu | |
| | | | | 440 | | | | | 445 | | | | | 450 | |
| Ile | His | Pro | Thr | Asp | Ser | Glu | Ser | Phe | Glu | | | | | | |
| | | | | 455 | | | | | 460 | | | | | | |

| 1990 | | 1991 | | 1992 | | 1993 | | 1994 | | 1995 | | 1996 | | 1997 | | 1998 | | 1999 | | 2000 | | 2001 | | 2002 | | 2003 | | 2004 | | 2005 | | 2006 | | 2007 | | 2008 | | 2009 | | 2010 | | 2011 | | 2012 | | 2013 | | 2014 | | 2015 | | 2016 | | 2017 | | 2018 | | 2019 | | 2020 | | 2021 | | 2022 | | 2023 | | 2024 | | 2025 | | 2026 | | 2027 | | 2028 | | 2029 | | 2030 | | 2031 | | 2032 | | 2033 | | 2034 | | 2035 | | 2036 | | 2037 | | 2038 | | 2039 | | 2040 | | 2041 | | 2042 | | 2043 | | 2044 | | 2045 | | 2046 | | 2047 | | 2048 | | 2049 | | 2050 | | 2051 | | 2052 | | 2053 | | 2054 | | 2055 | | 2056 | | 2057 | | 2058 | | 2059 | | 2060 | | 2061 | | 2062 | | 2063 | | 2064 | | 2065 | | 2066 | | 2067 | | 2068 | | 2069 | | 2070 | | 2071 | | 2072 | | 2073 | | 2074 | | 2075 | | 2076 | | 2077 | | 2078 | | 2079 | | 2080 | | 2081 | | 2082 | | 2083 | | 2084 | | 2085 | | 2086 | | 2087 | | 2088 | | 2089 | | 2090 | | 2091 | | 2092 | | 2093 | | 2094 | | 2095 | | 2096 | | 2097 | | 2098 | | 2099 | | 2100 | | 2101 | | 2102 | | 2103 | | 2104 | | 2105 | | 2106 | | 2107 | | 2108 | | 2109 | | 2110 | | 2111 | | 2112 | | 2113 | | 2114 | | 2115 | | 2116 | | 2117 | | 2118 | | 2119 | | 2120 | | 2121 | | 2122 | | 2123 | | 2124 | | 2125 | | 2126 | | 2127 | | 2128 | | 2129 | | 2130 | | 2131 | | 2132 | | 2133 | | 2134 | | 2135 | | 2136 | | 2137 | | 2138 | | 2139 | | 2140 | | 2141 | | 2142 | | 2143 | | 2144 | | 2145 | | 2146 | | 2147 | | 2148 | | 2149 | | 2150 | | 2151 | | 2152 | | 2153 | | 2154 | | 2155 | | 2156 | | 2157 | | 2158 | | 2159 | | 2160 | | 2161 | | 2162 | | 2163 | | 2164 | | 2165 | | 2166 | | 2167 | | 2168 | | 2169 | | 2170 | | 2171 | | 2172 | | 2173 | | 2174 | | 2175 | | 2176 | | 2177 | | 2178 | | 2179 | | 2180 | | 2181 | | 2182 | | 2183 | | 2184 | | 2185 | | 2186 | | 2187 | | 2188 | | 2189 | | 2190 | | 2191 | | 2192 | | 2193 | | 2194 | | 2195 | | 2196 | | 2197 | | 2198 | | 2199 | | 2200 | | 2201 | | 2202 | | 2203 | | 2204 | | 2205 | | 2206 | | 2207 | | 2208 | | 2209 | | 2210 | | 2211 | | 2212 | | 2213 | | 2214 | | 2215 | | 2216 | | 2217 | | 2218 | | 2219 | | 2220 | | 2221 | | 2222 | | 2223 | | 2224 | | 2225 | | 2226 | | 2227 | | 2228 | | 2229 | | 2230 | | 2231 | | 2232 | | 2233 | | 2234 | | 2235 | | 2236 | | 2237 | | 2238 | | 2239 | | 2240 | | 2241 | | 2242 | | 2243 | | 2244 | | 2245 | | 2246 | | 2247 | | 2248 | | 2249 | | 2250 | | 2251 | | 2252 | | 2253 | | 2254 | | 2255 | | 2256 | | 2257 | | 2258 | | 2259 | | 2260 | | 2261 | | 2262 | | 2263 | | 2264 | | 2265 | | 2266 | | 2267 | | 2268 | | 2269 | | 2270 | | 2271 | | 2272 | | 2273 | | 2274 | | 2275 | | 2276 | | 2277 | | 2278 | | 2279 | | 2280 | | 2281 | | 2282 | | 2283 | | 2284 | | 2285 | | 2286 | | 2287 | | 2288 | | 2289 | | 2290 | | 2291 | | 2292 | | 2293 | | 2294 | | 2295 | | 2296 | | 2297 | | 2298 | | 2299 | | 2300 | | 2301 | | 2302 | | 2303 | |
|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|------|--|
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